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CONTENTS OF VOLUME 120

No. 1, August, 1937

PAGE

Young, E. Gordon. On the separation and characterization of the proteins of egg white ... 1

Jones, Chase Breese, and du Vigneaud, Vincent. The synthesis of hexocystine and hexomethionine and a study of their physiological availability ... 11

Bergman, A. J., and Turner, C. W. The composition of rabbit milk stimulated by the lactogenic hormone ... 21

Graham, W. R., Jr., Houchin, O. B., and Turner, C. W. The production of urea in the mammary gland ... 29

Lichtman, A. L. Fatty acids and glucose in the blood of depancreatized dogs ... 35

Fenn, W. O., and Goettsch, Marianne. Electrolytes in nutritional muscular dystrophy in rabbits ... 41

Hoffman, William S. A rapid photoelectric method for the determination of glucose in blood and urine ... 51

Hoffman, William S. The photoelectric determination of potassium in minute quantities of serum ... 57

Burk, Norval F. Osmotic pressure, molecular weight, and stability of amandin and excelsin and certain other proteins ... 63

Morgan, Agnes Fay, Kimmel, Louise, and Hawkins, Nora C. A comparison of the hypervitaminoses induced by irradiated ergosterol and fish liver oil concentrates ... 85

Alving, Alf S., and Gordon, Wayne. Studies of urea, creatinine, and ammonia excretion in dogs in acidosis ... 103

Dobriner, Konrad. Porphyrin excretion in the feces in normal and pathological conditions ... 115

Stotz, Elmer, Harrer, Carter J., Schultze, M. O., and King, C. G. Tissue respiration studies on normal and scorbutic guinea pig liver and kidney ... 129

Jacobs, Walter A., and Gould, R. Gordon, Jr. The ergot alkaloids. XII. The synthesis of substances related to lysergic acid ... 141

Arnow, L. Earle. The formation of dopa by the exposure of tyrosine solutions to ultraviolet radiation ... 151

Schoenheimer, Rudolf, and Rittenberg, D. Deuterium as an indicator in the study of intermediary metabolism. IX. The conversion of stearic acid into palmitic acid in the organism ... 155

Bodansky, Aaron. Notes on the determination of serum inorganic phosphate and serum phosphatase activity ... 167

Barnard, Robert D. The reactions of nitrite with hemoglobin derivatives ... 177

Cori, Carl F., Cori, Gerty T., and Hegnauer, Albert H. Resynthesis of muscle glycogen from hexosemonophosphate ... 193

Rutenber, Charles B., and Andrews, James C. The applicability of the Benedict-Denis procedure to the determination of methionine sulfur ... 203

Schroeder, E. F., and Woodward, Gladys E. The enzymatic hydrolysis of glutathione by rat kidney ... 209

Mueller, J. Howard. Nicotinic acid as a growth accessory for the diphtheria bacillus ... 219

Venning, Eleanor Hill, Evelyn, Kenneth A., Harkness, E. V., and Browne, J. S. L. The determination of estrin in urine with the photoelectric colorimeter ... 225

Hogness, T. R., Sidwell, A. E., Jr., and Zscheile, F. P., Jr. The absorption spectra of compounds related to the sterols ... 239

Spencer, Howard C., Morgulis, Sergius, and Wilder, Violet M. A micromethod for the determination of gelatin and a study of the collagen content of muscles from normal and dystrophic rabbits ... 257

Lavietes, Paul H. Anaerobic ultrafiltration ... 267

Deuel, Harry J., Jr., Murray, Sheila, Hallman, Lois F., and Tyler, David B. Studies on ketosis. XII. The effect of choline on the ketonuria of fasting rats following a high fat diet ... 277

Butts, Joseph S., Blunden, Harry, and Dunn, Max S. Studies in amino acid metabolism. III. The fate of *dl*-leucine, *dl*-norleucine, and *dl*-isoleucine in the normal animal ... 289

Toennies, Gerrit. Relations of thiourea, cysteine, and the corresponding disulfides ... 297

Newton, Eleanor B. A chromogenic tungstate and its use in the determination of the uric acid of blood ... 315

No. 2, September, 1937

Leighty, John A., and Corley, Ralph C. Amino acid catabolism. IV. The fate of certain synthetic α-amino acids administered by subcutaneous injection to the normal dog ... 331

Sendroy, Julius, Jr. Microdetermination of chloride in biological fluids, with solid silver iodate. I. Gasometric analysis ... 335

Sendroy, Julius, Jr. Microdetermination of chloride in biological fluids, with solid silver iodate. II. Titrimetric analysis ... 405

Sendroy, Julius, Jr. Microdetermination of chloride in biological fluids, with solid silver iodate. III. Colorimetric analysis ... 419

Sendroy, Julius, Jr. Note on errors in the analysis of chloride in albuminous urine ... 441

Jacobs, Walter A., and Craig, Lyman C. The veratrine alkaloids. II. Further study of the basic degradation products of cevine ... 447

Harrison, Harold E. The sodium content of bone and other calcified material ... 457

NEUWIRTH, ISAAC. Sugar content of heparinized and oxalated plasmas. 463

BLOCK, RICHARD J. Chemical studies on the neuroproteins. II. The effect of age on the amino acid composition of human and mammalian brain proteins. 467

CAMPBELL, HAROLD A., and LINK, KARL PAUL. Derivatives of *d*-galacturonic acid. III. The synthesis of a mercaptal of *d*-galacturonic acid and aldehydo tetraacetylmethyl-*d*-galacturonate. 471

ROBINSON, HOWARD W., PRICE, J. WAIDE, and HOGDEN, CORINNE G. The estimation of albumin and globulin in blood serum. I. A study of the errors involved in the filtration procedure. 481

SCHOENHEIMER, RUDOLF, and JOHNSTON, CHARLES G. Lithocholic acid gallstones from hog bile. 499

RITTENBERG, D., SCHOENHEIMER, RUDOLF, and EVANS, E. A., JR. Deuterium as an indicator in the study of intermediary metabolism. X. The metabolism of butyric and caproic acids. 503

BRODIE, BERNARD B., and FRIEDMAN, MAX M. The determination of thiocyanate in tissues. 511

BEHRENS, OTTO K., and DU VIGNEAUD, VINCENT. The synthesis of anserine from *l*-1-methylhistidine. 517

RIEGEL, CECILIA, RAVDIN, I. S., and ROSE, HENRY J. Effect of bile with and without cholesterol esters on esterification of cholesterol in blood plasma. 523

STEKOL, JAKOB A., and HAMILL, WILLIAM H. On the non-labile deuterium of amino acids subjected to treatment in the medium of dilute deuterium oxide. 531

SULLIVAN, M. X., and HESS, W. C. The effect of aldehydes on the quantitative determination of cysteine and cystine. 537

PAPPENHEIMER, ALWIN M., JR. Diphtheria toxin. I. Isolation and characterization of a toxic protein from *Corynebacterium diphtheriæ* filtrates. 543

BODANSKY, OSCAR. The use of different measures of reaction velocity in the study of the kinetics of biochemical reactions. 555

LEVENE, P. A., and CHRISTMAN, CLARENCE C. On a catalytically induced reaction resembling the Cannizzaro reaction. 575

LEVENE, P. A., and KREIDER, LEONARD C. Oxidation and hydrolysis of polygalacturonide methyl ester to levo-tartaric acid. 591

LEVENE, P. A., and KREIDER, LEONARD C. The ring structure of α-methyl-*d*-galacturonide and its derivatives. 597

LEVENE, P. A., and TIPSON, R. STUART. The structure of monoacetone *d*-xylulose. 607

TIPSON, R. STUART. A note on the acridine salts of "yeast" and "muscle" adenylic acids. 621

REISER, RAYMOND. The lipid analysis of human thoracic duct lymph. 625

ALMQUIST, H. J. Further studies on the antihemorrhagic vitamin. 635

WRIGHT, NORMAN. The infra-red absorption spectra of the stereoisomers of cystine. 641

KAPLAN, A., and CHAIKOFF, I. L. The effect of choline on the lipid metabolism of blood and liver in the completely depancreatized dog maintained with insulin.................................... 647
YOUNG, LESLIE. The effect of pyocyanine on the metabolism of cerebral cortex....... 659
ARMSTRONG, W. D., and BREKHUS, P. J. Chemical constitution of enamel and dentin. I. Principal components................... 677
HARGER, R. N., HULPIEU, H. R., and LAMB, E. B. The speed with which various parts of the body reach equilibrium in the storage of ethyl alcohol.. 689
SCHMIDT, E. G., SCHMULOVITZ, M. J., SZCZPINSKI, A., and WYLIE, H. BOYD. The phenol and imidazole content of the blood.......... 705
MASON, HAROLD L., HOEHN, WILLARD M., MCKENZIE, BERNARD F., and KENDALL, EDWARD C. Chemical studies of the suprarenal cortex. III. The structures of Compounds A, B, and H 719
SHINOHARA, KAMENOSUKE. The determination of thiol and disulfide compounds, with special reference to cysteine and cystine. VIII. Molecular ratio between A-phospho-18-tungstic acid and cysteine in their color reaction................. 743
STEVENS, CHARLES D. The source of the formic acid produced on acid hydrolysis of nucleic acids................................ 751
LEVENE, P. A., ROTHEN, ALEXANDRE, and KUNA, MARTIN. Rotatory dispersion of configurationally related amines 759
LEVENE, P. A., ROTHEN, ALEXANDRE, and KUNA, MARTIN. The mechanism of the reaction of substitution and Walden inversion... 777
INDEX TO VOLUME 120 799

ON THE SEPARATION AND CHARACTERIZATION OF THE PROTEINS OF EGG WHITE

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(Received for publication, October 19, 1936)

The proteins of the white of hen's eggs other than albumin and ovomucoid have received little attention from investigators. Eichholz in 1898 first claimed the existence of ovomucin which he prepared by diluting egg white with 4 volumes of water. By acidifying the filtrate a further precipitate was formed which he called ovoglobulin. Osborne and Campbell (1900) confirmed the presence of ovomucin but denied the existence of a globulin. Recently Sörensen (1934) has again claimed the presence in egg white of both mucin and globulin by fractionation and her method of analysis of carbohydrate groupings. The properties of ovomucin have been studied only superficially and analysis has been limited to its elementary composition. The existence of ovoglobulin is open to question.

The purpose of the present investigation was to devise means of separating the various proteins and by analysis to determine their individuality with particular reference to the mucin-globulin complex. For the first time an effort has been made to separate and identify the protein of the chalazæ. The only reference to the chemistry of chalazæ in the literature is a paper by Liebermann (1888) in which duplicate analyses of C and H are recorded and N and S are stated to be present by qualitative tests.

The content of total nitrogen and of glucosamine is distinctive of mucoproteins. Cystine and, in confirmation, total sulfur have been determined as more distinctive of albumins and globulins. Out of the investigation as a whole have arisen broader considerations of the possibility of the separation of *natural* mixtures of proteins.

EXPERIMENTAL

Fractionation—Three series of preparations have been made on two general schemes of fractionation. The first depended on initial precipitation at half saturation of $(NH_4)_2SO_4$ and the second on dilution with distilled water. Preparation 1 was made by the first scheme and Preparations 2 and 3 by the second. All operations were carried out at approximately 0° with the exception of centrifuging. After any precipitation a period of 12 to 24 hours was allowed for completion and this also applied to each washing of such precipitates. When any fraction was ready for analysis according to the scheme of separation, it was dehydrated in alcohol and ether. All preparations resulted in snow-white, fine powders characteristic of this method of preparing proteins. All quantitative analyses were performed on this material. Physical properties were tested on the material before dehydration.

Scheme 1

The whites were separated from the yolks and the chalazæ and cooled to 0°. After thorough mixing the usual procedure of precipitating at half saturation of ammonium sulfate was followed. The filtrate was treated according to the procedure of Sörensen for the crystallization of ovalbumin. After three recrystallizations the material was dialyzed free from sulfate at 0°, precipitated in alcohol, and dehydrated as outlined above.

Mucin—The original precipitate was extracted repeatedly with a half saturated solution of $(NH_4)_2SO_4$ and redispersed partially in 2 per cent $(NH_4)_2SO_4$. On centrifuging, two layers were obtained; the upper a brilliantly opalescent limpid solution, the lower a thick gelatinous mass. The protein of the lower layer, presumably mucin, was not dispersed by KCl (1 per cent) or by HCl (1 per cent), showing atypical behavior. The material was extracted repeatedly with 0.2 per cent HCl (pH 2.9) until the washings were protein-negative, and with water until sulfate and chloride were negative.

Globulin—The protein of the upper layer was precipitated by raising the $(NH_4)_2SO_4$ concentration to one-third saturation. This material was readiy soluble in water and coagulable by heat. It was dialyzed free from sulfate and dehydrated.

Scheme 2

Dilution of the white with 2 to 3 volumes of water at 0° was found to precipitate all proteins precipitable by this means at pH 6.4.

Mucin—The precipitate had a glutinous character. It was washed with water and dispersed in NaCl (5 per cent), forming a gelatinous mass. The protein was again precipitated by dilution with 5 volumes of water. The precipitate was washed with NaCl (1 per cent) until protein-negative, with water till chloride-negative, and dehydrated.

Mucin Extract—The protein remaining in the dilute solution of NaCl was precipitated with alcohol and the precipitate washed free from chloride with 50 per cent alcohol. It was then dehydrated.

Globulin—The filtrate after the primary dilution should contain all albumin, mucoid, and possibly unprecipitated mucin. No precipitate could be obtained by further dilution or acidification to pH 5.0. A precipitate was formed on addition of an equal volume of $(NH_4)_2SO_4$ at pH 6.4. This material was readily soluble in water but left a slight gelatinous residue. The bulky precipitate was suspended and washed with half saturated $(NH_4)_2SO_4$ solution until the washing fluid no longer showed any protein reaction. The dissolution in water and reprecipitation by $(NH_4)_2SO_4$ was repeated. The precipitate was again dissolved in water and dialyzed free from sulfate as described under Scheme 1. Only a very small flocculent precipitate formed. This was centrifuged, washed repeatedly in water, and dehydrated.

Globulin Mother Liquor—The fluid in the dialyzer was still rich in protein. It was, therefore, prepared as a fraction by precipitation in alcohol and dehydrated.

Albumin—The filtrate from the precipitate by $(NH_4)_2SO_4$ was used for crystallization of albumin.

Mucoid—The remaining solution after centrifugation was coagulated in a boiling water bath for 30 minutes at pH 5. The filtrate was saturated with solid $(NH_4)_2SO_4$ and the mucoid precipitated. By this procedure the yield was low and in one case *no* mucoid was obtained. The precipitate was purified by dissolving in water, dialyzing to remove sulfate, and pouring into alcohol.

Chalazæ—The chalazæ were isolated from the combined whites and suspended in 5 per cent NaCl. They were extracted repeatedly until only a trace of protein could be detected in the supernatant fluid and then washed with distilled water till free from chloride. Under these conditions they appeared as small masses of jelly. After centrifugation they were dehydrated.

Analysis

All samples were first analyzed to determine the ash and volatile matter present. The values were generally small and in some cases no ash was present.

The total sulfur was determined by the gravimetric procedure of Stockholm and Koch (1923). The values for cystine were obtained by the Pollard-Chibnall (1934) modification of the Prunty modification of the Sullivan method.

The glucosamine was estimated by the colorimetric procedure suggested by Elson and Morgan (1933), glucosamine hydrochloride being used as standard. The figures obtained are mainly of comparative interest as a means to the identification of mucoprotein in the fractions. Hydrolysis was carried on with 5 per cent HCl on a boiling water bath for 2 hours and then by boiling directly for 1 to 2 hours longer.

The distribution of material among the different fractions was determined by weight in Preparation 3 after an estimation of the total protein in the mixed whites by the Kjeldahl method (see Table I).

The results obtained in a systematic analysis of the various fractions of the three preparations are summarized in Table II, expressed on a moisture- and ash-free basis.

After inspection of Table II it will be apparent that there is good agreement in the analysis of Preparations 2 and 3 which followed essentially the same procedure of fractionation.

The ovomucin fraction presents features characteristic of mucoproteins. The value for total N is low; the hexosamine content is high. The total S is of the order previously recorded for this group of proteins, *viz.* 1.7 per cent (Levene, 1925), and the cystine present accounts for about 70 per cent. The values obtained for cystine show the greatest discrepancies in this series. I have been unable to find in the literature any figures of the cystine content of mucoproteins for comparative purposes.

The figure for hexosamine content of 10 to 12 per cent is of the same order of magnitude as recent determinations of the carbohydrate radical in ovomucin and ovomucoid. Very few estimations on mucins have been made. Recent determinations are 12.8 per cent in gastric mucin, expressed as glucose, by the Hagedorn-Jensen method (Webster and Komarov, 1932); 14.9 per cent in ovomucin as mannose-galactose complex by the orcinol method (Sörensen, 1934); 12 to 15 per cent in umbilical cord mucin as reducing sugar (Fürth, Herrmann, and Scholl, 1934).

TABLE I

Distribution of Material among Different Fractions

	gm.	per cent
Total protein (N × 6.25)	155	100
Ovomucin	6.2	4.0
Protein of mucin extract	6.7	4.3
Ovoglobulin	0.2	0.1
Protein of globulin mother liquor	4.7	3.0
Ovomucoid	2.2	1.4

TABLE II

Analyses of Different Fractions

	Ovomucin			Mucin extract		Ovoglobulin			Mother liquor		Ovalbumin
Preparation No.	1	2	3	2	3	1	2	3	2	3	1
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Moisture	7.74	7.93	7.39	6.08	5.20	2.14	7.62	8.54	6.86	6.35	1.02
Ash	0.78	0.64	0.57		0.07	0.00			0.10	0.07	0.30
Total N*	11.8	12.6	13.1	13.7	14.3	18.9	14.3	14.8	14.5	14.3	14.4
" S*	1.59	1.80	1.80	0.77	0.79	1.80	1.08		0.89	0.92	1.47
Cystine*	3.25	5.61	4.84	2.35	2.36	<1	3.70	4.43	3.98	3.81	1.40
Glucosamine*	12.1	10.4	10.6	3.66	3.12	1.63	6.88	6.43	3.48	4.49	1.64

* On moisture- and ash-free basis.

When first precipitated the material has a fibrin-like elasticity but becomes more flocculent as electrolytes are washed away. It was found to be dispersed again partially or completely in basic solution such as 0.1 to 1.0 per cent NaOH and KOH, 1 per cent Na_2CO_3, saturated $Ba(OH)_2$, and also 4 per cent KCN, 50 per cent urea, 5 per cent NaCl, 6 per cent $K_3Na(SO_4)_2$. It is not dispersed by 2 per cent HCl and is not precipitated at the isoelectric point characteristic of mucins, pH 2.7 to 3. The analytical data suggest that ovomucin is a mucoprotein and an entity in egg white. Its solubility is anomalous. The first effort at precipitation, whether by dilution or salting-out at 0°, results in an elastic fibrinous mass impossible to redisperse to its previous natural state. The best solvents are those which redisperse denatured proteins and the solutions obtained are highly viscous. Further, the material on reprecipitation becomes more resistant to redispersion and an insoluble fraction remains. In general the ovomucin fraction behaves as if denaturation had taken place under the conditions of separation.

The fraction called mucin extract represents essentially the portion of the original mucin precipitate soluble in dilute saline. Analytically it was distinguished by a higher nitrogen content and lower cystine and hexosamine values. It did not behave as a globulin in that it was not precipitated on infinite dilution or on acidulation to pH 5.0. It was, however, precipitable on the addition of $(NH_4)_2SO_4$ to half saturation and was coagulable by heat. It is taken to be some occluded albumin and redispersed ovomucin.

The fraction called ovoglobulin represented a protein precipitated by $(NH_4)_2SO_4$ at half saturation and pH 6.4 and after dialysis. Preparation 1 was radically different from Preparations 2 and 3. Because of its high nitrogen and sulfur, it must have contained some ammonium sulfate. The other preparations were very similar to the protein which remained in solution in the dialyzer. The only difference was a higher hexosamine content in the precipitated protein. This fraction in all three preparations was very small in yield, being less than a gm. from 3 dozen eggs. This small yield in conjunction with the relatively high hexosamine content makes a separate identity very improbable. It is thus suggested that no globulin was present and that the material obtained on dialysis was traces of denatured mucin. It is also possible, however, that the primary material called in this paper ovomucin is a globulin, readily denatured and unusual in its content of nitrogen and carbohydrate.

The material remaining in aqueous solution in the dialyzer represented the mother liquor protein. This material was precipitated twice at half saturation of $(NH_4)_2SO_4$ and pH 6.4. It was quite similar in analysis to the globulin precipitate and differed in all points except nitrogen from the crystalline ovalbumin. The nature of this fraction presents difficulties in identification. It is not typical of globulin or albumin and requires further investigation. It may be albumin contaminated with some ovomucin.

The crystalline albumin was prepared merely for comparative purposes and as a check on the analytical methods. The percentage of nitrogen, 14.4, is lower than that usually accepted; as for example, 14.9 to 15.2 (Calvery and Titus, 1934). The value for cystine is of interest because of the particular modification of the colorimetric method used, and 1.40 per cent agrees well with recent determinations in the literature; *viz.*, 1.33 per cent (Calvery, 1931–32), 1.38 to 1.52 (Calvery and Titus, 1934), 1.3 per cent (Calvery, Block, and Schock, 1936), all determined by the Folin-Marenzi method, and 1.15 per cent (Sullivan and Hess, 1930) by the original procedure on which the Chibnall-Prunty modifications were based.

TABLE III

Comparison of Mucoprotein Preparations

	Ovomucin	Ovomucoid	Ovomucoid (Needham)	Chalasæ
	per cent	*per cent*	*per cent*	*per cent*
Moisture	7.69	4.67	3.02	8.58
Ash	0.66	0.52	0.50	
Total N*	12.5	12.60	12.7	13.3
" S*	1.73	1.36	0.89	1.08
Cystine*	4.57	3.95	4.10	4.10
Glucosamine*	11.0	9.70	14.0	11.4

* On moisture- and ash-free basis.

The value for glucosamine is also of some interest because of the uncertainty as to the presence of carbohydrate in pure albumins. By their orcinol method Sörensen and Haugaard (1933) have reported 1.71 per cent as mannose and Sörensen (1934) has confirmed this value, 1.7 per cent. Our figure of 1.64 per cent expressed as glucosamine is surprisingly close.

For comparative purposes with the various fractions removed ovomucoid was prepared as outlined above. Another specimen of ovomucoid was prepared by the method used by Needham (1927). The analyses of these preparations are given in Table III together with the average of the mucin analyses and that of the chalazæ.

In a comparison of the characteristics of these four samples there is a striking similarity. This is especially so between the ovomucoids and the substance of the chalazæ. In the latter the nitrogen is slightly higher. The glucosamine content is certainly suggestive of mucoprotein and the physical behavior is also indicative of this.

The determination of cystine in ovomucoid appears to be the first recorded. The estimation of the carbohydrate radical has been done very frequently. More recent determinations are 26.3 per cent (Izumi, 1925), 11.5 (Needham, 1927), 15 (Fürth, Herrmann, and Scholl, 1934) by reduction methods; and 7.4 (Tillmans and Philippi, 1929), 10 (Zuckerkandl and Messiner-Klebermass, 1931), 9.2 (Sörensen, 1934) by different color reactions. It may thus be concluded that there is a general agreement at least in the order of magnitude of the carbohydrate present of 10 to 15 per cent whether calculated as glucose or glucosamine.

The facts presented in this paper are of interest in themselves and in so far as they answer the problem of the separation of the protein constituents of egg white. The results suggest that we are creating artifacts in our efforts at separation by means of various solvents. The natural protein of egg white may be a mucoprotein-albumin complex broken by the tools which we use to separate it. It is worthy of ultracentrifugal analysis. The recent work of McFarlane (1935) on serum proteins with this type of analysis demonstrates the damaging effects on dispersion by dilution and by ammonium sulfate. Gortner and his coworkers (1928) have encountered similar difficulties of separation in the wheat flour proteins, as have McCalla and Rose (1935).

SUMMARY

The proteins of the white of hen's egg have been separated by two procedures into several fractions and compared on the basis

of their content of nitrogen, sulfur, cystine, and hexosamine. Ovomucin has been isolated by dilution and by $(NH_4)_2SO_4$ in a denatured form with total N at 12.5 per cent, total S 1.73, cystine 4.57, and hexosamine 11.0. No globulin has been found. The substance of the chalazæ has been identified as mucoprotein in nature.

The natural protein of egg white is suggested to be a mucoprotein-albumin complex separated into fragments dependent on the reagent employed and having no separate identity in egg white.

I wish to take this opportunity of thanking Sir Frederick Hopkins for the hospitality of the Sir William Dunn Institute during the summer of 1933, when this work was begun.

BIBLIOGRAPHY

Calvery, H. O., *J. Biol. Chem.*, **94**, 613 (1931–32).

Calvery, H. O., Block, W. D., and Schock, E. D., *J. Biol. Chem.*, **113**, 21 (1936).

Calvery, H. O., and Titus, H. W., *J. Biol. Chem.*, **105**, 683 (1934).

Eichholz, A., *J. Physiol.*, **23**, 163 (1898).

Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).

Fürth, O., Herrmann, H., and Scholl, R., *Biochem. Z.*, **271**, 395 (1934).

Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., *Cereal Chem.*, **6**, 1 (1928); Colloid symposium monograph, New York, **5**, 179 (1927).

Izumi, S., *Z. physiol. Chem.*, **142**, 175 (1925).

Levene, P. A., Hexosamines and mucoproteins, Monographs on biochemistry, London and New York, 126 (1925).

Liebermann, L., *Arch. ges. Physiol.*, **43**, 71 (1888).

McCalla, R. C., and Rose, R. C., *Canad. J. Research*, **12**, 346 (1935).

McFarlane, A. S., *Biochem. J.*, **29**, 660 (1935).

Needham, J., *Biochem. J.*, **21**, 733 (1927).

Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, **22**, 422 (1900).

Pollard, A., and Chibnall, A. C., *Biochem. J.*, **28**, 326 (1934).

Sörensen, M., *Biochem. Z.*, **269**, 271 (1934).

Sörensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933).

Stockholm, M., and Koch, F. C., *J. Am. Chem. Soc.*, **45**, 1953 (1923).

Sullivan, M. X., and Hess, W. C., *Pub. Health Rep.*, *U. S. P. H. S.*, suppl. 86 (1930).

Tillmans, J., and Philippi, K., *Biochem. Z.*, **215**, 36 (1929).

Webster, D. R., and Komarov, S. A., *J. Biol. Chem.*, **96**, 133 (1932).

Zuckerkandl, F., and Messiner-Klebermass, L., *Biochem. Z.*, **236**, 19 (1931)

THE SYNTHESIS OF HEXOCYSTINE AND HEXOMETHIONINE AND A STUDY OF THEIR PHYSIOLOGICAL AVAILABILITY

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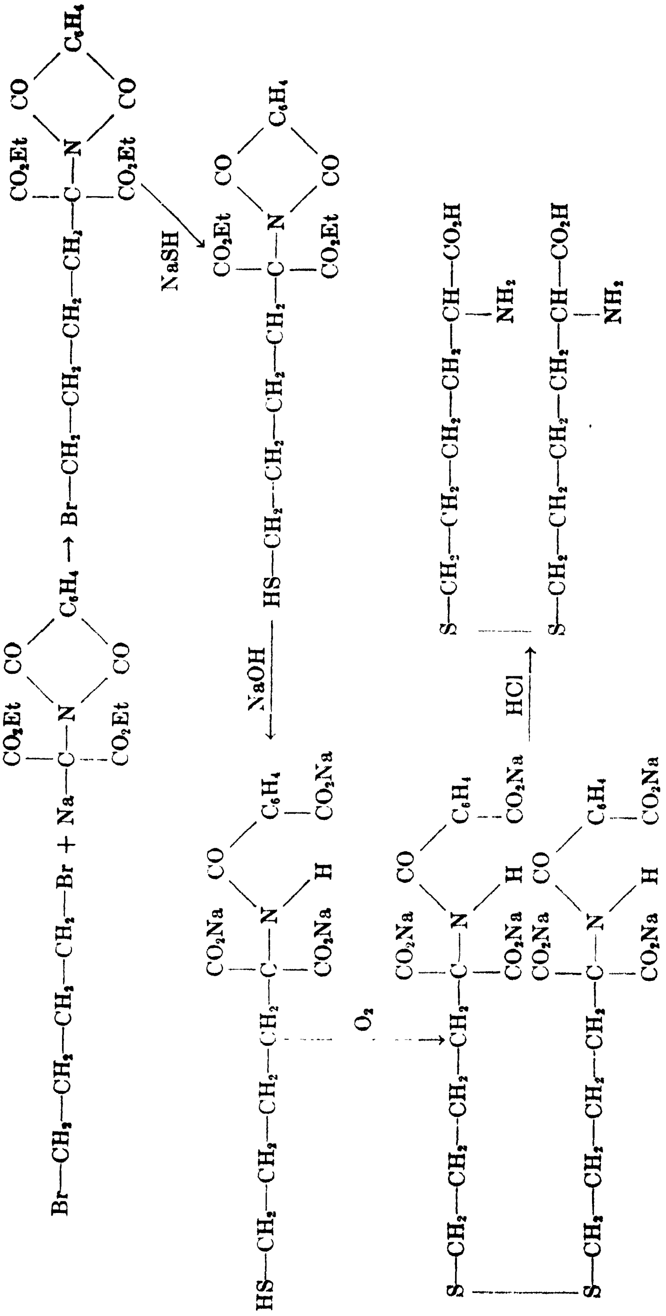
(Received for publication, May 25, 1937)

In a previous investigation the growth-promoting power of pentocystine was studied in order to see if the ability of homocystine to support growth of animals on a cystine-deficient diet in lieu of cystine was shared by other disulfide amino acids (1). Homomethionine, the S-methylpentocysteine, was also included in the study from like considerations with respect to the relationship of methylthiol amino acids to methionine. The negative results reported for pentocystine and homomethionine demonstrated that the ability to substitute for cystine in the diet is not a general property of either disulfide or methylthiol amino acids. There remained, however, the possibility that even though pentocystine and homomethionine were not capable of supporting growth, the next higher homologues of these two amino acids might possess this property. The disulfide of the 6-carbon chain homologue might conceivably be converted to homocystine in the body, and hence serve in lieu of cystine, whereas that of the 5-carbon chain homologue might not be convertible either to homocystine or directly to cystine. The same considerations applied as well to homomethionine and its next higher homologue. We felt that, before we could consider completed this phase of our study concerning the availability of the higher homologues of homocystine and methionine, the 6-carbon homologues should be investigated.

The present communication deals with the synthesis and growth-promoting powers of bis-ϵ-amino-ϵ-carboxypentyl disulfide, which is the disulfide of the 6-carbon homologue of cysteine,


Br—CH₂—CH₂—CH₂—CH₂—Br + Na—C(CO₂Et)₂—N(CO)₂C₆H₄ → Br—CH₂—CH₂—CH₂—CH₂—C(CO₂Et)₂—N(CO)₂C₆H₄
NaSH
HS—CH₂—CH₂—CH₂—CH₂—C(CO₂Et)₂—N(CO)₂C₆H₄
NaOH
HS—CH₂—CH₂—CH₂—CH₂—C(CO₂Na)₂—N(H)—CO—C₆H₄—CO₂Na
O₂
HCl
S—CH₂—CH₂—CH₂—CH₂—CH(NH₂)—CO₂H
S—CH₂—CH₂—CH₂—CH₂—CH(NH₂)—CO₂H

and ε-methylthiol-α-aminocaproic acid, which is the homologue of methionine containing a chain of 6 carbon atoms. For convenience we shall designate these compounds as hexocystine and hexomethionine respectively.

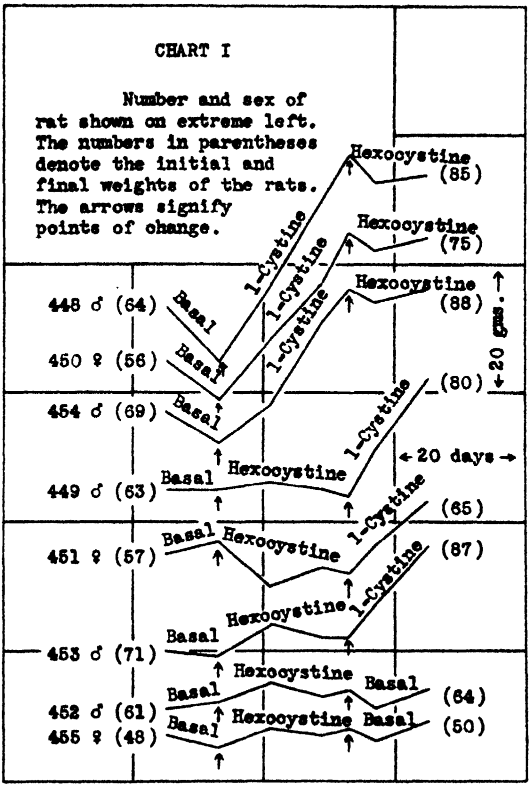


Chart I. Growth of animals on a cystine-deficient diet supplemented with hexocystine.

The synthesis of hexocystine was effected by methods analogous to those used for the synthesis of pentocystine. The accompanying series of reactions was involved.

The hexomethionine was prepared from the hexocystine by reduction of the latter in liquid ammonia by sodium followed by the addition of methyl iodide to the liquid ammonia solution. Various derivatives of both amino acids were prepared and analyzed to confirm the structures assigned to them.

The hexocystine and hexomethionine were fed to animals maintained on a cystine-deficient diet, and, as shown in Charts I and II, neither of these compounds was able to support growth under these conditions. Further and more conclusive evidence has, therefore, been added to that already obtained from our studies with pentocystine and homomethionine that the ability of homocystine and methionine to serve in lieu of cystine in supporting growth is not a general property of either disulfide or methylthiol amino acids. The evidence points all the more to the unique relationship of methionine and homocystine to cystine.

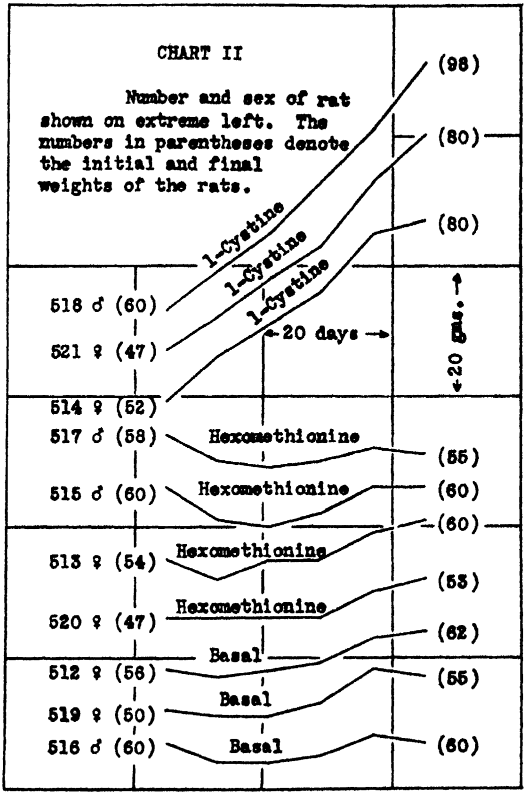


Chart II. Growth of animals on a cystine-deficient diet supplemented with hexomethionine.

It might be of interest to point out that hexocystine is isomeric with ϵ,ϵ'-diaminodi(α-thio-n-caproic acid) which was recently synthesized by Greenstein (2) for comparison with cystine in physical chemical studies of multivalent amino acids. The present synthesis of hexocystine makes available a very interesting compound for comparison with Greenstein's isomer from this same standpoint.

EXPERIMENTAL

Preparation of Hexocystine—Diethyl succinate was prepared by esterification of succinic acid according to the method of Thielepape (3). To a solution of 400 gm. of succinic acid in 2 liters of absolute alcohol were added 1 liter of benzene and 1 cc. of concentrated H_2SO_4. The solution was refluxed for 18 hours in such a way that the vapors passed through calcium carbide to remove the water formed in the esterification. 496 gm. of ester were obtained, representing 84 per cent of the theoretical yield.

The diethyl succinate was reduced catalytically under high pressure (4) to the corresponding glycol through the courtesy of Dr. Homer Adkins of the University of Wisconsin. Tetramethylene bromide was then obtained by passing HBr into the glycol at 80° (5). From 60 gm. of tetramethylene glycol 108 gm. of the bromide were obtained.

Diethyl-γ-bromobutylphthalimidomalonate was prepared by the condensation of the tetramethylene bromide with diethyl sodium phthalimidomalonate. 130 gm. of the bromide and 30 gm. of the sodium phthalimidomalonic ester were heated at 160–165° for 4 hours under a reflux condenser fitted with a $CaCl_2$ tube. The excess tetramethylene bromide was removed by distillation *in vacuo* and finally by steam distillation. About 80 gm. of the tetramethylene bromide were recovered.

For the introduction of the sulfhydryl group into the molecule treatment with NaSH was employed. An aqueous solution of NaSH was prepared by passing H_2S into 30 cc. of a 40 per cent solution of NaOH until the reaction was no longer alkaline to phenolphthalein (6). The diethylbromobutylphthalimidomalonate was dissolved in 500 cc. of 95 per cent ethyl alcohol. To this solution was added the aqueous NaSH solution and the mixture was allowed to stand overnight. It was then refluxed for 1 hour on a steam bath and the alcohol was distilled off under reduced pressure in an atmosphere of nitrogen. The residue was repeatedly extracted with ether in the presence of about 500 cc. of 10 per cent HCl. The ether was removed from the combined ether extracts by distillation. Saponification of the syrupy residue was effected by refluxing it for 2 hours with 150 cc. of 5 N NaOH.

To the alkaline solution, HCl was added until the reaction was just acid to phenolphthalein. 2 drops of 5 per cent $FeCl_3 \cdot 6H_2O$ were added and the solution was aerated until the nitroprusside test for sulfhydryl was negative.

The above solution was diluted to 700 cc., 100 cc. of concentrated HCl were added, and the solution was refluxed in an oil bath at 120–130° for 2 hours. 500 cc. more of concentrated HCl were added and the solution was heated for 45 minutes longer. The hydrolysate was then concentrated almost to dryness *in vacuo,* and the phthalic acid and inorganic salt were moved by filtration whenever necessary during the distillation.

The crude hexocystine hydrochloride was purified in the following manner. The moist residue from the above concentration was dissolved in about 50 cc. of water, heated to boiling with a small amount of carbex E, cooled, and filtered. The hexocystine was then precipitated by careful neutralization to litmus with 5 N NaOH. After the mixture had been allowed to stand for 30 minutes in an ice bath, it was filtered and the precipitate was washed with water. This was then dissolved in 50 cc. of dilute HCl, treated twice with carbex E, and reprecipitated. This process was repeated and the final precipitate was washed successively with small portions of water, alcohol, and ether. The yield of the final pure product, dried first in the air and then in a vacuum desiccator over P_2O_5, was 6.6 gm., or 44 per cent of the theoretical yield, based on the amount of diethyl sodium phthalimidomalonate used. The compound crystallized in microrosettes and decomposed at 273–276° (corrected). Like pentocystine, hexocystine gives a negative sulfhydryl and positive disulfide test, a positive ninhydrin reaction, and a negative Sullivan test for cystine. For analysis the compound was dried *in vacuo* over P_2O_5 at 110°. It had the following composition.

$C_{12}H_{24}O_4N_2S_2$. Calculated, N 8.64, S 19.77; found, N 8.68, S 20.05

Preparation of S-Benzylhexocysteine—2.0 gm. of hexocystine were added to 30 cc. of liquid ammonia. Metallic sodium was added until a very slight excess was indicated by the persistence of the blue color. 2.2 cc. of benzyl chloride were added dropwise and the ammonia was allowed to evaporate spontaneously at room temperature. The residue was dissolved in 25 cc. of water, filtered, and the filtrate made just acid to litmus with HCl. The precipitate was filtered and was dissolved in 25 cc. of dilute HCl. This solution was diluted to 500 cc. and was extracted with ether. The aqueous layer was neutralized and the precipitated S-benzylhexocysteine was filtered and was recrystallized from water. 2.5 gm. of the purified product were obtained, representing 81 per cent of the theoretical yield. It melted with decomposition at 240–242° (corrected) and crystallized in platelets. The product for analysis was dried over P_2O_5 at 100° *in vacuo* and had the following composition.

$C_{13}H_{19}O_2NS$. Calculated, N 5.53; found, N 5.56

Preparation of N-Formyl-S-Benzylhexocysteine—2.0 gm. of S-benzylhexocysteine were dissolved in 15 cc. of 85 to 90 per cent formic acid. The solution was warmed to 60° and 5 cc. of acetic anhydride were slowly added with shaking. The solution was kept at 60° for 30 minutes, then cooled, and 5 cc. of water were added. The solution was concentrated to dryness *in vacuo*. The residue was dissolved in 10 cc. of acetone, and 20 cc. of benzene were added. This solution was concentrated to 15 cc. and the crystalline N-formyl-S-benzylhexocysteine was filtered. The final product melted at 103–104° (corrected) and appeared as colorless plates. The compound after being dried over P_2O_5 at 61° *in vacuo* had the following composition.

$C_{14}H_{19}O_3NS$. Calculated, N 4.98; found, N 5.05

Preparation of Hexomethionine—3.0 gm. of hexocystine were added to 40 cc. of liquid ammonia and metallic sodium was added until a blue color just persisted. 1.5 cc. of methyl iodide were then added dropwise. After evaporation of the ammonia, the residue was dissolved in 10 cc. of water. The solution was filtered and was neutralized to litmus with 10 per cent HI. 4 volumes of absolute ethyl alcohol were added and the solution was filtered after being cooled in an ice bath. An additional crop of crystals was obtained by concentrating the mother liquors and alcohol washings to about 5 cc. and adding 4 volumes of absolute alcohol. The products were recrystallized from a minimum amount of hot water. The total yield of thin plates amounted to 2.8 gm., or 85 per cent of the theoretical value. The compound melted with decomposition at 276–278° (corrected). For analysis the compound was dried *in vacuo* over P_2O_5 at 100° and had the following composition.

$C_7H_{15}O_2NS$. Calculated, N 7.91, S 18.09; found, N 8.02, S 17.98

Preparation of Benzenesulfonylhexomethionine—The method employed for the preparation of this compound was that described by Gurin and Clarke (7) for the corresponding derivative of methionine. To a solution of 0.5 gm. of hexomethionine in 2.3 cc. of N NaOH, 1.2 gm. of benzenesulfonyl chloride and 13.6 cc. of N NaOH were slowly added with shaking. The solution was then shaken for 4 hours, filtered, and acidified to Congo red. The crystals which formed on chilling the solution were filtered and were recrystallized from water. The dried needle-like crystals melted at 86–87° (corrected) and had the following composition.

$C_{13}H_{19}O_4NS_2$. Calculated, N 4.41, S 20.21; found, N 4.43, S 20.20

Growth Experiments

The growth-promoting properties of hexocystine and of hexomethionine were tested by the administration of these compounds to young white rats upon a cystine-deficient diet. The same procedure which has been used in similar studies in this laboratory was followed (8) and the details will therefore not be given here. The basal diet had the following composition: casein 5.0, dextrin 34.0, sucrose 15.0, agar 2.0, salt mixture (Osborne and Mendel (9)) 4.0, lard 19.0, cod liver oil 5.0, and milk vitamin concentrate (Supplee *et al.* (10)) 16.0.

Eight rats from the same litter were used for the investigation of hexocystine. They were placed on the basal diet for 8 days. At the end of this time to each of five rats were given daily two dextrin pills each containing 54 mg. of hexocystine and 200 mg. of dextrin. Three rats were given two dextrin pills daily, each pill containing 10 mg. of *l*-cystine. The hexocystine fed was 4 times the equivalent of the *l*-cystine supplement. After 20 days the rats that had received *l*-cystine were given the hexocystine supplement in lieu of the cystine. Three of the rats which had been given hexocystine were given *l*-cystine. The other two animals were given no supplement. The food consumption is recorded in Table I and the growth curves are given in Chart I.

TABLE I

Food Consumption

Rat No. and sex	Days	Supplement	Daily food consumption	Rat No. and sex	Days	Supplement	Daily food consumption
			gm.				*gm.*
448 ♂	1- 8		5.7	453 ♂	1- 8		5.9
	8-28	*l*-Cystine	7.9		8-28	Hexocystine	7.8
	28-40	Hexocystine	8.2		28-40	*l*-Cystine	7.6
450 ♀	1- 8		4.5	452 ♂	1- 8		4.9
	8-28	*l*-Cystine	7.6		8-28	Hexocystine	6.7
	28-40	Hexocystine	6.4		28-40		7.3
454 ♂	1- 8		4.9	455 ♀	1- 8		6.0
	8-28	*l*-Cystine	7.3		8-28	Hexocystine	7.1
	28-40	Hexocystine	6.8		28-40		7.2
449 ♂	1- 8		5.2	518 ♂	1-40	*l*-Cystine	7.8
	8-28	Hexocystine	7.2	521 ♀	1-40	"	6.4
	28-40	*l*-Cystine	9.0	514 ♀	1-40	"	6.9
				517 ♂	1-40	Hexomethionine	5.5
451 ♀	1- 8		5.4	515 ♂	1-40	"	5.8
	8-28	Hexocystine	5.6	513 ♀	1-40	"	6.0
	28-40	*l*-Cystine	7.9	520 ♀	1-40	"	5.2
				512 ♀	1-40		6.3
				519 ♀	1-40		5.6
				516 ♂	1-40		6.2

A litter of ten rats was used for the study of hexomethionine. Four rats were given the basal diet plus 60 mg. of hexomethionine and three rats were given the basal diet plus 20 mg. of *l*-cystine daily. Three animals were maintained on the basal diet alone. The hexomethionine supplement corresponded to twice the sulfur content of the *l*-cystine supplement. The hexomethionine and *l*-cystine were given in the form of dextrin pills as in the hexocystine experiments. The amount of food consumed is given in

Table I. Chart II presents the growth curves obtained in the experiments concerned with hexomethionine.

SUMMARY

The synthesis of the disulfide of the 6-carbon homologue of cysteine, bis-ϵ-amino-ϵ-carboxypentyl disulfide, and of the second higher homologue of methionine, ϵ-methylthiol-α-aminocaproic acid, has been presented. These compounds have been designated as hexocystine and hexomethionine respectively.

S-Benzylhexocysteine, N-formyl-S-benzylhexocysteine, and benzenesulfonylhexomethionine have also been prepared.

It has been shown that neither hexocystine nor hexomethionine is capable of supporting growth of young animals on a cystine-deficient diet, thus affording further evidence that the ability of homocystine and methionine to serve in lieu of cystine in the diet is not a general property of disulfide and methylthiol amino acids.

BIBLIOGRAPHY

1. Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **108**, 73 (1935).
2. Greenstein, J. P., *J. Biol. Chem.*, **109**, 529 (1935).
3. Thielepape, E., *Ber. chem. Ges.*, **66**, 1454 (1933).
4. Adkins, H., and Folkers, K., *J. Am. Chem. Soc.*, **53**, 1095 (1931).
5. Müller, A., *Monatsh. Chem.*, **49**, 27 (1928).
6. Schütz, F., *Angew. Chem.*, **46**, 780 (1933).
7. Gurin, S., and Clarke, H. T., *J. Biol. Chem.*, **107**, 395 (1934).
8. Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **109**, 477 (1935).
9. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
10. Supplee, G. C., Kahlenberg, O. J., and Flanigan, G. E., *J. Biol. Chem.*, **93**, 705 (1931).

THE COMPOSITION OF RABBIT MILK STIMULATED BY THE LACTOGENIC HORMONE*

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The studies on the composition of milk experimentally stimulated by the lactogenic hormone are very limited. Catchpole, Lyons, and Regan (1) stimulated the secretion of milk in virgin Holstein heifers. In one case 500 cc. of secretion per day were produced. Analysis showed that the fat content, pH, and coagulation time were within the usual limits of variation. The taste was salty owing to low lactose and high chloride content. As it has been shown by Turner (2) that virgin heifers may be induced to produce considerable amounts of secretion by its regular removal, the part played by the hormone injection is in doubt.

Evans (3) reported the copious production of milk in virgin dairy goats with lactogen. It was stated that the preliminary observations indicate that the first milk secreted under these experimental conditions strongly resembled colostrum (globulin fractions, casein, percentage of total nitrogen).

Grant (4) reinitiated lactation in multiparous guinea pigs which had been allowed to involute for several days until only small amounts of lactose-free serous fluid were being produced. The secretion subsequently stimulated by the injection of lactogen was analyzed for lactose and total protein. The small amount of secretion produced resembled milk in its physical appearance but possessed an abnormally low lactose content (0.04 to 0.24 per cent).

In none of the observations on the composition of milk induced as a result of the injection of the lactogenic hormone are the results entirely free of objection. In virgin animals, lactogen can

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induce only duct lactation, whereas in normal parturient animals the secretion of milk comes chiefly from the alveolar epithelium. Further, the secretions produced should be compared with colostrum rather than with normal milk, unless young are adopted by the animal in which the lactation has been experimentally stimulated and nursing is continued for a number of days before milk samples are taken.

Incidentally, it may be of interest to report that female rabbits in which lactation was experimentally initiated were maintained in lactation without further hormone administration for periods of 30 to 37 days by using suckling rabbits 6 to 10 days old. Similarly, a male rabbit induced to lactate raised three young to a weaning age. Unfortunately, samples of milk from these animals were not obtained.

The object of the present paper is to present the results of two studies concerned with the influence of the lactogenic hormone upon the secretion of milk by the rabbit.

The first experiment was concerned with a study of the lactose content of the entire mammary gland of the rabbit in various stages of lactation. As lactose is a synthetic product of the mammary gland and is not present in the "dry" gland (Campbell (5)), it appeared that the amount of lactose present in the gland would be a good measure of the activity of the lactogenic hormone in initiating lactation. Gardner and Turner (6) had earlier suggested the use of rabbits pseudopregnant for 12 to 16 days as suitable assay animals for this hormone. For increasing degrees of response four ratings had been suggested. 1+ indicated merely duct lactation, 2+ indicated that the ducts were distended and the lobules slightly milky, 3+ indicated full lactation, and 4+ maximum distension of the gland.

In the first experiment individual glands of a number of rabbits which had been induced to lactate experimentally were removed, the whole gland was ground in a mortar with washed sand, and the tissue extracted three times with water, centrifuged, and the supernatant fluid decanted into 50 or 100 ml. volumetric flasks. A protein-free filtrate was obtained by mixing 8 volumes of the above solution with 1 volume each of $ZnSO_4$ and NaOH. The glucose was then removed by fermentation with washed yeast at 32° for 15 minutes. The lactose was then determined by means of the Shaffer-Somogyi (7) copper-iodometric sugar technique.

The lactose content of the glands rated 1+ varied from 0.05 to 0.12 per cent, the 3+ glands 0.60 per cent, and the 4+ glands from 0.87 to 0.96 per cent lactose (Table I).

In a second experiment a series of five rabbits was injected with 2 ml. daily of the same lactogenic preparation and then killed at intervals of 2 days and a series of glands removed from the right and left sides for individual analysis. The average lactose content of the glands was found to increase until the 6th day, then declined. This appears to indicate that when the level of injection of the lactogenic hormone is inadequate to bring the gland to a 4+ rating, the continued injection at that level will not cause further development. Considerable variation is to be noted in the lactose content of individual glands. There does not seem to be a tendency for any particular glands in the series to show more secretory activity than others, although, on the average, the front glands contained slightly more lactose. Comparison of the average lactose values of the right and left series of glands showed remarkable uniformity (Table II).

TABLE I

Lactose Content of Rabbit Mammary Glands

Rabbit No.	Position of glands removed*	Gardner-Turner assay	Lactose content of entire gland
			per cent
1	Left, 1	+	0.09
	" 4	+	0.10
6	" 1	+	0.06
	Right, 2	+	0.05
7	Left, 1	+	0.05
	Right, 2	+	0.06
9	" 2	+	0.09
11	" 1	+	0.12
	" 2	+	0.07
10	Left, 3	++?	0.13
8	" 1	+++	0.60
2	" 1	++++	0.87
3	" 1	++++	0.92
4	" 1	++++	0.96
5	Right, 4	++++	0.98

* Glands are numbered from front to rear.

Considering all the observations on the lactose content of the glands with the ratings assigned, it would appear that glands rated 1+ contain from about 0.05 to 0.14 per cent, 2+ glands contain from about 0.15 to 0.35 per cent, 3+ glands contain from about 0.36 to 0.75 per cent, and 4+ glands from 0.76 to 1.00 per cent of lactose. While there are one or two ratings which would be changed by the chemical analysis of the glands, it is doubtful whether the accuracy which would be attained by the analysis of single glands would be sufficient to warrant use of that method over mere visual inspection of the gland.

TABLE II

Variation in Lactose Content of Glands of Rabbits

Rabbit No.	Side	Length of injection	Gardner-Turner assay	Lactose content of glands from front to rear					Average lactose content
				1	2	3	4	5	
		days		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
12	Left	2	+	0.08	0.09		0.10	0.11	0.09
	Right	2	+	0.06	0.13		0.10	0.10	0.10
13	Left	4	+++(Poor)	0.74	0.52		0.48	0.45	0.54
	Right	4	+++ "	0.81	0.61	0.37	0.40	0.43	0.56
14	Left	6	+++	0.59	0.42		0.80	0.70	0.64
	Right	6	+++	0.78	0.50	0.56	0.62	0.69	0.63
15	Left	8	+++?	0.26	0.46		0.38	0.13	0.34
	Right	8	+++?	0.35	0.33		0.38	0.18	0.32
16	Left	10	++	0.26	0.30		0.29	0.36	0.30
	Right	10	++	0.22	0.14		0.31	0.31	0.25
Average....				0.42	0.35		0.39	0.35	
20	Left	6	++	0.27	0.37		0.27	0.38	0.32
	Right	6	++	0.29	0.23		0.35	0.42	0.31

The object of the second study was to compare the composition of rabbit milk obtained at various intervals after normal parturition with the milk obtained from pseudopregnant rabbits in which lactation had been induced experimentally by the injection of the lactogenic hormone for 6 days. The milk was removed by suction and the manual compression of the gland. In all cases pituitrin (Parke, Davis and Company) in amounts of 0.05 to 0.10 ml. was injected intravenously to aid in expressing the milk.

Methods of Milk Analysis

In most cases the standard method of milk analysis was used. Specific gravity was determined by means of a pycnometer at 15.6°; total solids by drying in an oven at 90–92° for 24 hours or until the sample ceases to lose weight; fat by the Babcock test; total nitrogen by the Kjeldahl method; casein by diluting the milk with 9 volumes of water at 40–42° and precipitating with 10 per cent acetic acid and determining the nitrogen by the Kjeldahl method; albumin by neutralizing the filtrate from casein with NaOH, adding 10 per cent acetic acid, and heating on a water bath until the albumin was precipitated, then filtered, and the nitrogen determined as for casein; ash by drying the sample on a water bath and then ashing below redness. Lactose was determined by precipitating the proteins with $Zn(OH)_2$ and determining the amount of sugar present in the filtrate by using the Shaffer-Somogyi (7) copper-iodometric sugar technique.

DISCUSSION

While the observations are not extensive, it appears that the most significant change in the transition of rabbit milk from colostrum to normal milk is in the protein fraction. Even 4 days after parturition the milk appears to approach normal composition. The analyses of Folin *et al.* (8) are quite similar to our sample of normal milk. The higher protein content of the milk reported by Pizzi (9) leads us to think that it might have been colostrum (Table III).

The samples of milk obtained from the rabbits induced to lactate experimentally by means of the lactogenic hormone compare rather well with the samples of colostrum. The lactose content of the milk from the 4+ rabbit is perfectly normal. As the rating declines, the lactose content of the gland declines also, just as it did when the entire glands were analyzed. Considering these data from the point of view of the mechanism of lactose synthesis, it would appear that the lactogenic hormone was capable of stimulating the synthesis of lactose in practically normal amounts when lactation equal to that observed following normal parturition was obtained. The increasing amounts of lactose in the secretion as full lactation is attained might be advanced as evidence in favor of the theory that the mechanism of lactose synthesis is the limiting factor in milk secretion.

TABLE III

Composition of Normal and Experimentally Induced Rabbit Milk

Milk sample No.	Sp. gr.	Fat	Total protein	Casein	Albumin-globulin	Lactose	Ash	Total solids	Remarks
Colostrum									
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
22	1 053	14 40	15 78	11 00	4 14	1 71	2 60	36 37	After parturition
23	1 045		16 74						After parturition
Semicolostrum									
10	1.038	17 81	10 63	7 20	2 34	1 83	1 94	33 73	4 days after parturition
Normal milk									
11	1 041	12 17	10 38	6 24	3 18	1 84	2 00	26 61	9 days after parturition
From literature									
	1 049	10.45	15 54			1.95	2 56	30 50	Pizzi (1894)
		12 10	11 40			1.80			Folin *et al.* (1919), average of 19 samples
Lactation induced in pseudopregnant rabbits by lactogenic hormone									
2		20 61				1.80	1.65	37.19	++++
21	1.046	12 47	13 44	9.93	3 06	1.43	1 67	28 80	+++
3		16 86				1 29	1.84	37 94	+++
7						0 93			+++
20						0.86			++ (Strong)

The fat content of the "experimental" milk varies within the normal range, except the first sample of 20.61 per cent. The ash content appears to be slightly below normal. In general it appears that milk approaching that secreted by normal parturient rabbits can be stimulated in pseudopregnant rabbits by the use of the lactogenic hormone. It should be noted that the lactogenic hormone preparations used in these experiments were not highly purified and probably contained other pituitary principles.

SUMMARY

In a study of the lactose content of the mammary glands of rabbits in which various degrees of lactation were stimulated experimentally with the lactogenic hormone, it was found that glands which were rated 1+ according to the method of Gardner and Turner contained from 0.05 to 0.14 per cent of lactose in the fresh gland. The 2+ glands contained from about 0.15 to 0.35 per cent, the 3+ glands about 0.36 to 0.75 per cent, and the 4+ glands from 0.76 to 1.00 per cent of lactose.

A comparison was made between the composition of milk secreted by rabbits after parturition with the milk experimentally initiated in pseudopregnant rabbits by the lactogenic hormone. Comparison of colostrum with the "experimental" milk in a 4+ rabbit showed that the lactose and total solids were similar; the fat, however, was higher and the ash content lower in the "experimental" milk. It was found that the amount of lactose in the milk increases as the ratings of the glands increase.

BIBLIOGRAPHY

1. Catchpole, H. R., Lyons, W. R., and Regan, W. M., *Proc. Soc. Exp. Biol. and Med.*, **31**, 301 (1933).
2. Turner, C. W., *Missouri Agric. Exp. Stat., Research Bull. 156* (1931).
3. Evans, E. I., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1372 (1933).
4. Grant, G. A., *Biochem. J.*, **30**, 2027 (1936).
5. Campbell, J. A., *Quart. J. Exp. Physiol.*, **7**, 53 (1913).
6. Gardner, W. U., and Turner, C. W., *Missouri Agric. Exp. Stat., Research Bull. 196* (1933).
7. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
8. Folin, O., Denis, W., and Minot, A. S., *J. Biol. Chem.*, **37**, 349 (1919).
9. Pizzi, A., *Staz. sper. agr. ital.*, **26**, 615 (1894); *Exp. Stat. Rec.*, **6**, 668 (1894).

THE PRODUCTION OF UREA IN THE MAMMARY GLAND*

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The sole site of formation of urea in the animal body is generally accepted as being in the liver. Van Slyke and Cullen (1) found that during the digestion of protein in the dog, the urea content of the blood passing through the liver increased by approximately 2 mg. per cent. Lower levels of urea were found elsewhere in the systemic blood, particularly in the renal veins. These results have been confirmed by London (2), who showed that the liver elaborates urea continuously, the rate of synthesis simply increasing with the digestion of proteins and the consequent assimilation of amino acids by the blood. Van Slyke and Cullen (1) found no evidence from their blood analyses of formation of urea in the muscles.

Bollman, Man, and Magath (3) showed that when the liver and the kidneys were removed from an animal the blood urea level remained constant. On the other hand, if only the kidneys were removed, the level of urea in the blood rose hour by hour.

During the course of investigations on the nutrition of the mammary gland by the circulating blood, there was occasion to determine the urea content of the arterial blood and mammary venous blood of goats during lactation soon after parturition. The technique used in these experiments was comparable to that used by London *et al.* in their work on the liver. The precautions outlined by Graham, Kay, and McIntosh (4) for sampling bloods in the study of lactation were carefully observed.

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The animals used in the experiments had been previously surgically prepared by amputation of one mammary gland. All efferent vessels except the mammary vein were tied and cut in the remaining gland, thus giving complete drainage through the mammary vein. The arterial samples were taken from the carotid artery which had been exteriorized previously.

Urea was determined on whole blood samples by the urease method of Van Slyke (5). Table I shows typical findings picked from rough data at random for urea for duplicate samples of blood. These results indicate that the maximum error of the method in our hands was of the order of 1.6 per cent. The average error was of the order of 0.6 per cent between duplicates.

TABLE I

Duplicate Values for Urea Nitrogen Found in Arterial and Mammary Venous Bloods, together with Recalculation of Venous Figures to Maximum Theoretical Concentration

Arterial	Error	Mammary	Error	Mammary blood as corrected for water loss
mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
25.87	0.10	27.49	0.34	27.21
25.97		27.83		27.55
26.39	0.43	27.56	0.12	27.28
26.82		27.68		27.40
28.07	0.02	29.18	0.03	28.84
28.09		29.21		28.91
18.00	0.18	19.87	0.17	19.67
18.18		19.70		19.50
32.65	0.11	33.62	0.14	33.28
32.76		33.76		33.42
Average . . . 26.28	0.17	27.59	0.16	27.31
% error	0.65		0.58	

The results of analyses of several arterial and mammary bloods taken simultaneously are shown in Table II and indicate that there is a higher level of urea in the mammary venous blood than in the arterial blood. The four figures that show exception to this general conclusion are in bloods taken between 1 and 2 hours after the animals had been milked. The average values from all experiments show that the venous bloods contain 3.2 per cent more urea than the arterial bloods. Since the four bloods taken 1 to 2 hours after milking appear as a group to be physiologically different from the remaining bloods, it seems justifiable to class the bloods taken later in the milking period in one group. In this case the average increase in the venous level of urea N amounts to 4.5 per cent of the arterial level.

TABLE II

Levels for Urea Nitrogen Found in Simultaneous Samples of Arterial and Mammary Venous Bloods of Goats in Early Lactation

Goat No.	Level of urea N found		Arterial minus mammary	Remarks
	Arterial	Mammary		
	mg. per cent	*mg. per cent*	*mg. per cent*	
403	20.23	20.75	−0.52	Nursing young
30	15.84	17.17	−1.33	
403	15.10	14.91	+0.19	1 to 2 hrs. after milking
348	16.30	15.30	+1.00	
30	22.06	21.62	+0.44	
403	18.90	18.92	−0.02	
348	28.08	29.98	−1.90	4 hrs. after milking
30	32.70	33.69	−0.99	
403	21.19	22.00	−0.81	
348	29.65	30.94	−1.29	8 " " "
403	21.57	22.94	−1.37	
30	31.43	31.50	−0.07	
348	19.74	20.97	−1.23	14 " " "
30	26.60	27.62	−1.02	
403	18.12	19.78	−1.66	
Average of all samples........	22.50	23.21	−0.71	= 3.2% increase in venous level
Average of 4, 8, 12 hr. samples.....	25.45	26.60	−1.15	= 4.5% increase in venous level

A higher level of a substance in the venous blood than is found in the arterial blood might be accounted for by the concentration of the blood on its passage through the mammary gland, the water lost being excreted for milk formation. Blackwood and Stirling (6), Lintzel (7), and Graham, Jones, and Kay (8) were unable to demonstrate water losses that would affect significantly these analytical findings. Their concentration calculations were based on the analyses of the bloods for iron hemoglobin and cell volume. The results of four hemoglobin comparisons made during the present work showed that the amount of water lost by the bloods could not account for the differences found in urea. The loss of water was less than 1 per cent. Similarly, in other unpublished experiments, the amount of water withdrawn from the blood, as calculated from the blood volume flow by the thermostromuhr method of Herrick and Baldes (9), and the milk yield indicate that the withdrawal of water for the formation of milk is not more than 1 per cent of the whole blood volume. Increasing the venous blood figures by this amount and calculating the error as outlined above still leave significant amounts of urea to be accounted for. The difference found, therefore, cannot be due to concentration of the venous bloods.

The possibility of the storage and release of a substance as readily diffusible as urea seems to be unlikely, even in the tissues of an organ showing the peculiarities of the mammary gland. If we may assume that storage may be ruled out, it would appear that the higher levels of urea found in the venous blood as it comes from the mammary gland are due to the formation of urea within that organ.

The results of the experiments given are interpreted as showing that the mammary gland can produce urea from some nitrogenous source. It is a well known fact that high protein feeding produces a stimulation of lactation beyond that point at which protein itself appears to be a factor limiting the formation of the milk proteins. Gowen and Tobey (10) and Gaines (11) have suggested that the formation of lactose is *a*, if not *the*, major factor in limiting milk volume yield. The apparent positive relationship between the arterial-venous differences for blood sugar and the lactation yield of cows found by Graham *et al.* (8) might be taken as evidence in support of this theory. The source of the nitrogen forming the urea produced by the mammary glands is undoubtedly indirectly from the feed protein. Our knowledge of the deaminization mechanism of this type of metabolism in the liver presents the possibility of carbohydrate forming by-products of the reaction, if amino acids are the source of urea N. Thus the ability of the mammary gland to form urea may throw some new light on the stimulating effect of high protein diets on lactation.

BIBLIOGRAPHY

1. Van Slyke, D. D., *Arch. Int. Med.*, **19**, 56 (1917).
2. London, E. S., Kotschneff, N., Cholopoff, A., Abaschidze, T. S., and Alexandry, A. K., *Arch. ges. Physiol.*, **219**, 238 (1928).
3. Bollman, J. L., Mann, F. C., and Magath, T. B., *Am. J. Physiol.*, **69**, 371 (1924).
4. Graham, W. R., Jr., Kay, H. D., and McIntosh, R. A., *Proc. Roy. Soc. London, Series B*, **120**, 319 (1936).
5. Van Slyke, D. D., *J. Biol. Chem.*, **73**, 695 (1927).
6. Blackwood, J. H., and Stirling, J. D., *Biochem. J.*, **26**, 357 (1932).
7. Lintzel, W., *Z. Zucht., Reihe B*, **29**, 219 (1934).
8. Graham, W. R., Jr., Jones, T. S. G., and Kay, H. D., *Proc. Roy. Soc. London, Series B*, **120**, 330 (1936).
9. Herrick, J. F., and Baldes, E. J., *Physics*, **1**, 407 (1931).
10. Gowen, J. W., and Tobey, E. R., *J. Gen. Physiol.*, **15**, 45, 67 (1931-32).
11. Gaines, W. L., *Holstein-Friesian World*, **33**, 579 (1936).

FATTY ACIDS AND GLUCOSE IN THE BLOOD OF DEPANCREATIZED DOGS

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Murlin and Lusk (1, 2) demonstrated in their calorimeter experiments that, following glucose ingestion by the normal dog, carbohydrate may be utilized in preference to fat and that fat may be synthesized from carbohydrate. A fall in blood fat might accompany the former condition or a rise the latter. McClure and Huntsinger (3) have reported that, after the feeding of dextrose or protein as well as fat to normal humans, the blood fat rose about 100 mg. per 100 cc. in each case.

The elevated blood fat of partially depancreatized dogs has been linked with the deficiency of the internal secretion of the pancreas (4). A gradual rise in the blood fatty acids following pancreatectomy has been demonstrated in dogs (5). Since the totally depancreatized animal derives almost all its energy from the incomplete oxidation of fat (6), it is important to know how the changes in blood fatty acids are related to the rapid rise in blood sugar immediately following pancreatectomy. When the hypophysis is removed in addition to the pancreas, the low level of ketone body excretion indicates that a more complete oxidation of fat occurs in such animals (7). Furthermore, the rise in the respiratory quotient after glucose ingestion (8) by some hypophysectomized-depancreatized (Houssay) dogs and the elevated basal quotients have recently been correlated with a low level of glucose oxidation (9). Therefore, in these experiments certain phases of the relation between carbohydrate and fat metabolism have been studied by comparing simultaneous determinations of the blood sugar and fatty acids in the normal, depancreatized, and Houssay dog after the administration of glucose.

The nephelometric method for the determination of fat in the blood used by McClure and Huntsinger (3) and Bloor, Gillette, and James (4) and the modified method of Stewart and White used by Himwich *et al.* (5) have undergone criticism, such as to warrant reexamination with a more accurate method.

The fatty acid method employed here was a modification of the plan of Stoddard and Drury made by Man and Gildea (10). In view of the comments of Stewart and Hendry (11), oxidation of unsaturated fatty acids was prevented by passing a stream of CO_2 over the refluxing mixture and by the addition of 0.1 cc. of 0.1 per cent alcoholic hydroquinone during saponification. Table I shows recoveries of known amounts of oleic and stearic acids added to aliquot portions of a sample of dog serum. Sodium amytal was chosen as the anesthetic for the pancreatectomies after it was shown that intraperitoneal injections of 55 to 65 mg. per kilo in control animals produced a gradual fall in the fatty acids of only 10 mg. per 100 cc. in 5 hours. Samples of blood were taken from the femoral artery of docile animals and the fatty acids extracted from the serum within ½ hour.

TABLE I

*Recovery of Oleic and Stearic Acids**

Amount added		Observed titer	Amount recovered	
Oleic acid	Stearic acid			
m.-eq.	*m.-eq.*	*m.-eq.*	*m.-eq.*	*per cent*
0	0	12.23		
0	0	12.17		
10.00	0	21.86	9.66	96.6
10.00	0	22.01	9.81	98.1
5.00	0	17.00	4.80	96.0
5.00	0	17.13	4.93	98.6
0	10.00	21.73	9.53	95.3
0	10.00	21.79	9.59	95.9
0	5.00	17.16	4.94	98.8
0	5.00	17.10	4.90	98.0
10.00	10.00	31.58	19.38	96.9
10.00	10.00	31.84	19.64	98.2

* Added to dog serum containing 12.20 milli-equivalents of fatty acids per liter.

Effect of Removal of Pancreas (*Table II*)—The dogs weighed between 11 and 23 kilos and were in a postabsorptive state after a standard diet. Complete pancreatectomy caused a rise in the fatty acids which began 5 to 7 hours after the gland was removed. The blood sugar began to rise rapidly during the 1st postoperative hour, as has been shown by Chambers and Coryllos (12). Three dogs showed a significant fall in the blood fatty acids 1 hour after the gland was removed. This may be due to blood volume changes or the effect of a high blood sugar with adequate insulin still available in the tissues. In Dog 197 hypophysectomy preceded pancreatectomy by 1 month. Before the pancreatectomy the blood fatty acids were low and the sugar normal. Removal of the pancreas caused a much slower rise in the blood fatty acids and sugar than pancreatectomy with the hypophysis intact.

TABLE II

Effect of Complete Pancreatectomy on Blood Sugar and Fatty Acids

rs. after peration	Dog 1		Dog 2		Dog 3		Dog 4		Dog 197*		Dog 6†			
											1st stage		2nd stage	
	F.a.	G.	F.a.	G.	F.a.	G.	F.a.	G.	F.a.	G.	F.a.	G.	F.a.	G.
0	351	89	336	93	315	83	369	75	192	96	299	92	264	179
1	309	170	298	162	310	93	350	144	244	90	310	120	253	237
3	340	216	324	240	322	181	371	209	237	78	277	131	256	253
5	417	269	357	285	325	201	385	260	289	83	285	166	250	262
7	465	295	393	291	330	208	598	278	280	128	294	177	285	309
9	524	321	434	276	379	243	531	295	295	153	299	174	349	316
24			638	301	498	255			375	236				
30					490	284			445	283				

F. a. = fatty acids in mg. per 100 cc. of serum; G. = glucose in mg. per 100 cc. of whole blood.

* Hypophysectomized 1 month previous to the pancreatectomy.

† First operation: nine-tenths of gland removed, one-tenth transplanted subcutaneously with its circulation intact. Second operation: subcutaneous transplant removed under local anesthetic.

In lieu of a control laparotomy, the pancreas of Dog 6 was removed in two stages by the technique of Hédon. First the pancreas was detached from the duodenum and one-tenth of the gland with its circulation intact was pocketed subcutaneously in the abdominal wall. This caused little immediate change in the fatty acids, while the blood sugar rose steadily. 4 days later, with the fatty acids low and the sugar high, the remaining pancreatic tissue was removed under local anesthesia. This caused a further rise in the blood sugar, and the fatty acids began to increase after the 7th hour. The results following simultaneous cholecystectomy and pancreatectomy in one dog did not differ from those following simple pancreatectomy.

TABLE III

Effect of Ingestion of 50 to 70 Gm. of Glucose in 150 Cc. of Water

Dog No.	Glucose ingested		Hrs. after glucose ingestion					
			0	1	3	5	7	9
	gm. per kg.							
Normal								
9	2 2	F.a.	331	292	262	314	336	326
		G.	81	149	70	91	77	79
10	3 4	F.a.	350	356	283	310	327	342
		G.	76	119	91	80	81	75
11	3 5	F.a.	379	366	317	358	362	384
		G.	79	102	73	76	74	76
Depancreatized								
3	4 8	F.a.	421	430	396	419	419	430
		G.	279	501	480	508	429	413
12	3 6	F.a.	517	521	531	525	521	528
		G.	211	386	325	299	256	279
13	3 3	F.a.	449	453	466	455	472	458
			330	563	551	399	356	342
Houssay								
197	3 2	F.a.	413	418	409	412	418	416
		G.	252	520	681	538	420	350
199	5 0	F.a.	605	602	620	601	605	611
		G.	273	483	769	411	337	325
201	3 0	F.a.	370	365	365	368	369	365
		G.	210	377	433	403	378	310

F.a. = fatty acids in mg. per 100 cc. of serum of arterial blood; G. = glucose in mg. per 100 cc. of whole blood.

Fourteen determinations on six Houssay dogs at intervals up to 53 days postoperative showed fatty acid levels between 379 and

605 mg. per 100 cc., with occasional visible hyperlipemia, and blood sugar levels between 210 and 310 mg. per 100 cc.

Effect of Ingestion of 50 to 70 Gm. of Glucose (*Table III*)—Normal dogs were in a postabsorptive condition after a standard diet. Depancreatized and Houssay dogs would not accept the standard diet and were given 500 to 800 gm. of meat, 40 gm. of carbohydrate, a few gm. of fat, and pancreatin. The depancreatized dogs received insulin[1] up to 64 hours before the experiment. In three normal dogs the fatty acid concentration of the blood was reduced 17 to 20 per cent (60 to 70 mg. per 100 cc. of serum) at the end of the 3rd hour following the ingestion of 50 to 70 gm. of glucose by stomach tube. A fall in the hemoglobin from 13.2 to 12.8 gm. per 100 cc. during the 3rd hour was not sufficient to explain the fall in the fatty acids on the basis of blood dilution due to the glucose absorption. In the diabetic and Houssay dogs there was no significant fall in the fatty acids resulting from the ingestion of glucose, although the typically high and prolonged hyperglycemia occurred in both groups.

DISCUSSION

After the removal of the pancreas the blood fatty acids do not begin to rise until the blood sugar level is about 240 to 300 mg. per 100 cc. Blix (13), in his clinical study of diabetic lipemia, noted the pronounced lipemia of sera whose sugar content was over 200 mg. per 100 cc. The delayed rise in sugar after a partial pancreatectomy or pancreatectomy after hypophysectomy is accompanied by a delayed rise in fatty acids. When the blood sugar is between 100 and 200 mg. per 100 cc. and some insulin is probably still present, the fat is low or normal. Here the cells of the body are presented with large amounts of glucose which they can utilize; in this respect the condition resembles that which exists in the normal dog after the ingestion of glucose. These determinations following pancreatectomy indicate that cessation or slowing of the chain of events in glucose oxidation is followed by an increase in the fatty acids in the blood, brought about by mobilization from the depots, fulfilling a greater need of the cell for fat. A rise in the rate of oxidation of glucose is accompanied by a fall in the blood fatty acids.

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[1] Eli Lilly and Company kindly supplied the insulin.

In the normal dog the ingestion of glucose caused a fall in the blood fatty acids which was at a maximum in 3 hours, whereas such a sparing action on fat was absent in the depancreatized and Houssay dogs. Direct correlation of these results with glucose oxidation is not possible, but it is of interest to note that calorimeter experiments on the three Houssay dogs showed no rise in R.Q. for 4 hours after the ingestion of glucose. In one of the animals (Dog 199) quotients of 0.78 were obtained in balance studies with meat and glucose. This indicated a low level of carbohydrate oxidation (9). Although differing in this respect from the depancreatized dogs, the levels of the blood fatty acids were about the same in both groups.

SUMMARY

Removal of the pancreas in dogs causes an immediate rise in the blood sugar, while the blood fatty acids begin to increase 5 to 7 hours later.

Ingestion of 50 to 70 gm. of glucose causes a fall in the total fatty acids in the blood of normal dogs. This sparing action on fat is absent in the depancreatized and Houssay dogs.

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BIBLIOGRAPHY

1. Murlin, J. R., and Lusk, G., *J. Biol. Chem.*, **22**, 15 (1915).
2. Lusk, G., *J. Biol. Chem.*, **20**, 555 (1915).
3. McClure, C. W., and Huntsinger, M. E., *J. Biol. Chem.*, **76**, 1 (1928).
4. Bloor, W. R., Gillette, E. M., and James, M. S., *J. Biol. Chem.*, **75**, 61 (1927).
5. Himwich, H. E., Chambers, W. H., Hunter, A., and Spiers, M., *Am. J. Physiol.*, **99**, 619 (1932).
6. Barker, S. B., Chambers, W. H., and Dann, M., *J. Biol. Chem.*, **118**, 177 (1937).
7. Houssay, B. A., *New England J. Med.*, **214**, 971 (1936).
8. Biasotti, A., *Rev. soc. argentina biol.*, **10**, 82 (1934); *Compt. rend. Soc. biol.*, **116**, 898 (1934).
9. Chambers, W. H., Sweet, J. E., and Chandler, J. P., *Proc. Am. Physiol. Soc., Am. J. Physiol.*, **119**, 286 (1937).
10. Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, **99**, 43 (1932–33).
11. Stewart, C. P., and Hendry, E. B., *Biochem. J.*, **29**, 1677 (1935).
12. Chambers, W. H., and Coryllos, P., *Am. J. Physiol.*, **78**, 278 (1926).
13. Blix, G., Studies on diabetic lipæmia, Lund (1925).

ELECTROLYTES IN NUTRITIONAL MUSCULAR DYSTROPHY IN RABBITS

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Goettsch and Pappenheimer (1931) have described a nutritional muscular dystrophy in rabbits and guinea pigs fed on a diet (Diet 11) composed chiefly of grains and milk products. A somewhat similar pathological condition of the muscles has been observed by the same authors (unpublished results) in rabbits on a grain diet. Synthetic diets of casein, corn-starch, yeast, salts, cod liver oil, and cellophane were found by Madsen, McCay, and, Maynard (1935) to induce the same disorder. There is evidence that the onset of these muscle lesions is prevented or delayed by the addition of vegetable oil to the diet (Madsen, 1936; Morgulis and Spencer, 1936, *a*; and Pappenheimer and Goettsch, 1936). Rabbits with nutritional muscular dystrophy offer an excellent opportunity for the study of the electrolyte distribution in pathological muscles.

Method

Analyses for potassium, sodium, chloride, magnesium, and calcium were made in the Rochester laboratory with the assistance of Miss Doris M. Cobb. The rest of the work was done at the College of Physicians and Surgeons (by M. G.). (We are indebted to Dr. A. M. Pappenheimer of the Department of Pathology, Columbia University, for his interpretation of the pathology of the muscles studied.)

For moisture, the samples were dried for 20 hours at 100°. For creatine, the muscle was hydrolyzed and the creatinine determined according to the method of Rose, Helmer, and Chanutin

(1927). For total phosphorus, the dried muscle was incinerated with $MgNO_3$ and the phosphate determined colorimetrically by the Fiske and Subbarow (1929) method.

For chloride, the dried samples were soaked in hot water for 3 hours as recommended by Sunderman and Williams (1931) and analyzed by the method of Van Slyke (1923–24). For potassium, sodium, calcium, and magnesium, the samples were removed by hot 1 M HCl from the vials in which they were dried and rinsed into platinum crucibles. After evaporation to dryness on a steam bath, the samples were ashed in an electric furnace for 15 hours at 450–500°. The ash was dissolved in hot, concentrated hydrochloric acid because of insoluble phosphates and was analyzed for sodium by the method of Salit (1932), and for potassium by the method of Shohl and Bennett (1928) with some minor modifications (Fenn and Cobb, 1935). Calcium was precipitated as the oxalate at pH 5.0 (Peters and Van Slyke (1932) p. 769) and determined by Van Slyke and Sendroy's manometric method (Peters and Van Slyke (1932) p. 421). In Rabbits 278, 287, and 291, larger amounts of calcium were expected and accordingly the microtitration method with 0.01 N permanganate was used (Peters and Van Slyke, p. 767). In the precipitation and washing of the calcium, the improvements suggested by Wang (1935) were adopted. The magnesium in the supernatant fluid was precipitated as phosphate (Peters and Van Slyke (1932) p. 786) and determined colorimetrically by the method of Fiske and Subbarow (1929). The results are expressed in milli-equivalents per kilo of fresh tissue.

Results

The analyses were carried out on white muscle from three normal rabbits, four rabbits on Diet 11,[1] six on the grain mixture,[2] three on the grain mixture with 2 per cent cod liver oil, and one rabbit on a synthetic diet.[3] Most of the pathological animals were killed during the last stages of prostration due to muscular weakness.

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[1] Diet 11 consisted of rolled oats, 35.5 parts; wheat bran, 18.0; skim milk powder, 27.5; casein, 7.5; lard, 8.0; cod liver oil, 1.0; NaCl, 1.0; $CaCO_3$, 1.5.

[2] The grain mixture consisted of rolled oats, 32.5 parts; ground barley, 42.5; ground wheat, 22.5; and wheat bran, 2.5.

[3] We are indebted to Dr. L. L. Madsen for this muscle. The diet and history of the rabbit have been published (Madsen, 1936).

The results of the analyses are shown in Table I. The muscles were arbitrarily graded from + to ++++ according to (1) the severity of the microscopic lesions as described previously by Goettsch and Pappenheimer (1931) and Goettsch and Brown (1932), and (2) the degree of calcification shown by the fibers. None of these muscles presented fatty replacement.

All of the potassium figures and many of the other figures (indicated in Table I) are averages of at least two determinations on duplicate samples from the same muscle. The average difference between eight pairs of duplicate creatine determinations was 8 per cent, and the average difference between twenty-one pairs of duplicate potassium determinations was about 3 per cent. This error includes the error of sampling and is not purely analytical.

A few of the pathological muscles showed enormous amounts of calcium which, in Rabbits 210 and 226, were not exactly determined by the method employed because insufficient oxalate was used to secure complete precipitation. In later analyses this was avoided. These high values confirm the presence of calcium deposits observed microscopically.

In muscles with high calcium content, the total phosphorus was also increased, presumably indicating deposits of tricalcium phosphate. However, when dystrophy was present without calcification, the muscles were somewhat low in this constituent. This finding is in accordance with other observations which will be published elsewhere. Low values for some of the phosphorus fractions in this type of muscle have been reported by Morgulis and Spencer (1936, *b*). The large amounts of total phosphorus and of calcium found in some of the muscles are difficult to include in a total electrolyte balance and they have accordingly been omitted from the summary in Table II.

The presence of calcified fibers may account for some of the wide variations in the amounts of creatine which were lost from the various degenerated muscles. Goettsch and Brown (1932) reported a decrease in the muscle creatine of adult rabbits during nutritional muscular dystrophy which was roughly proportional to the the severity of the lesions. Degenerated muscle from rabbits

TABLE I

Electrolytes in Normal and Pathological Rabbit Muscles

	Rabbit No.	Time on diet	Muscle	Pathological examination		H_2O	M.-eq. per kilo fresh tissue						
				Severity of lesion	Extent of calcification		Creatine	Ca	P* (total)	K†	Mg	Na	Cl
		days				*cc. per kg.*							
Normal diet	254		Gluteus	–	–	754	41.2	2.0	(73.1 Average)	116.0	26.7	30.7	11.4
	255		"	–	–	772	38.5	1.7		122.2	24.6	17.0†	10.8
	256		"	–	–	765	35.2	1.8		116.1	24.2	34.1	11.3
Diet 11, adult rabbits	206	327	" ‡	++	++	757	33.4	10.6		95.2	22.5	53.8	25.4
			Gastrocnemius	++++	+++	745	24.2	235.5		88.8	§	79.0	35.4
	229	353	Gluteus	+++	–	783	20.6	6.4	58.9†	74.7	14.8	79.3	44.6
	191	628	"	+++	–	775	15.0†	21.9	60.5	80.6	17.0	68.6	46.0
	207	448	"	+++	–	785	14.6†	5.3	60.6†	57.5	13.4	81.3	47.6
Grain diet, adult rabbits	223	94	"	+	–	801	16.7	4.3†	62.6	53.5	16.3†	70.9	49.5
	227	187	" ‡	++	++	801	29.2†	48.8†	76.8	78.9	20.1†	58.8	32.0
	200	105	Gluteus	++	+	769	46.9†			82.8			
			Pectoralis	++	+	780	35.8†			78.0	18.2	68.7	35.4
			Gastrocnemius	+++	+	757	35.6†			79.0			
	210	120	Gluteus	+++	+++	761	27.9†	390+	213†	60.9		69.9	39.3
	234	76	"	+++	+	768	29.7	115.5†	108†	75.9	§	66.2	34.6
	226	60	" ‡	++++	++++	757	24.8	600+	322	54.3		77.8	28.6†

Grain diet with cod liver oil, 2%, young rabbits	278	67	Quadriceps	+++	−	793	24.0	11.4	84.0	97.7	18.4		
	287	32	"	+++	−	803	28.3	8.6	77.3	93.1	16.8		
	291	25	"	+++	+++	793	24.2†	385.0	178.7	86.4	28.6		
Synthetic diet, young rabbit	M2	14	Gluteus	++++	+	749	28.0†	69.0†	101†	103.3	§	76.2	28.8

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* Assumed to be monovalent. The normal P figure in parentheses is an average from thirty-six similar muscles.

† Average of two or more analyses.

‡ The gluteus was analyzed but not examined pathologically; the severity of lesions and extent of calcification were judged from other muscles of the rabbit.

§ High values of 25 to 35 milli-equivalents per kilo were obtained in these muscles but they are omitted because of the high calcium found in the same muscles. It was feared that the oxalate added might have been insufficient to precipitate all the calcium, as it certainly was in Rabbits 210 and 226.

on the grain diet did not show this marked change: sometimes the creatine remained normal; sometimes it fell to concentrations characteristic of muscle of rabbits subjected to prolonged inanition (Myers and Fine, 1913). In these muscles calcified fibers were found more frequently than in dystrophic muscle induced by Diet 11. In rabbits with muscle dystrophy on the grain diet, calcification of the muscle was recorded in twelve among thirteen adults (92 per cent) and twelve among nineteen young animals (63 per cent). On Diet 11, seven of forty-one diseased adult rabbits (17 per cent) and five of forty-one young ones (12 per cent) presented calcified lesions. It is seen in Table I that in dystrophic muscle from adult rabbits the creatine content is low unless calcification is present, in which case the creatine may remain nearly normal. The records of other rabbits substantiate this relationship. From Table I it is also apparent that the relationship between low creatine and absence of calcified fibers does not hold in the case of young rabbits. The creatine concentration of normal and pathological muscles has been found to vary even more widely in young rabbits than in adults, and therefore no generalization is attempted.

TABLE II

Summary

Diet	H_2O	M.-eq. per kilo fresh tissue									
		K	Mg	Na	Cl	Creatine	Δ K	Δ Mg	Δ Na	Δ Cl	Δ Creatine
	cc. per kg.										
Normal...	764	118.7	25.2	22.9	11.2	38.3	0	0	0	0	0
Diet 11...	766	79.3	16.9	72.4	39.8	21.5	−39.4	−8.3	+49.5	+28.6	−16.8
Grain.....	774	67.4	18.2	68.7	35.4	31.6	−51.3	−7.0	+45.8	+24.2	−6.7

With the exceptions mentioned, all the results in Table I are consistent with the general finding that in nutritional dystrophy of rabbits, the cellular elements are decreased in amount, as indicated by the decrease in potassium, phosphorus, magnesium, and creatine; while the extracellular tissue is correspondingly increased, as indicated by the increase in sodium and chloride. The evidence that in normal muscles sodium and chloride are located chiefly outside the muscle fibers in the interstitial fluid or connective tissue cells has recently been summarized (Fenn, 1936).

A reliable total electrolyte balance is hardly possible from the data at hand, but a good idea of its main features can be derived from the summary in Table II which shows the average variation from normal electrolyte content found in two series of pathological muscles (Diet 11 and grain diet). The gain in sodium (in equivalents) is found to be nearly twice as great as the gain in chloride. Evidently some of the sodium gained is not in the interstitial fluid with the chloride, where the Na:Cl ratio must be at least as low as in blood (1.3). This gain in sodium not accounted for by chloride is more than balanced by the loss in potassium and magnesium. This extra cation loss is in turn balanced by the loss in soluble phosphate reported by Morgulis and Spencer (1936, *b*) in similar muscles. Hence it may be concluded that the sodium gained in excess of chloride went into the cells in exchange for potassium or magnesium, and the potassium and magnesium which did not exchange with sodium came out as phosphate.

The loss in creatine confirms the previous finding of Goettsch and Brown (1932). The loss in potassium is strikingly greater in equivalent amount than the loss in creatine. The potassium loss cannot, therefore, be explained on the basis of the splitting up of a hypothetical undissociated potassium-phosphocreatine compound unless free creatine is retained for some other reason.

It is important to note that the sums of the sodium, potassium, and magnesium contents of the three series of rabbits are nearly equal. Thus the cation equivalents (without calcium) in Table II are 167, 169, and 154, respectively, in the normal animals, those on Diet 11, and those on the grain diet. Differences may be partly accounted for by varying amounts of soluble calcium. In any event, the agreement is close enough to rule out the possibility that the electrolyte changes observed were due to infiltration of fat which was not chemically determined. Microscopic sections of the muscle showed no excess of interstitial adipose tissue, and the water content also remained fairly constant.

The figures in Table II have been interpreted as indicating that some of the fibers have disintegrated so that their contents have taken on the composition of the interstitial fluids. In support of this interpretation, a calculation may be made of the volumes of the fibers on the basis of the potassium, magnesium, chloride, and creatine figures (Table III). In this calculation, it is assumed that chloride is wholly outside the fibers (in a concentration equal to that in the plasma), and potassium, magnesium, and creatine wholly inside (in a concentration equal to that in normal muscle fiber). If the chloride in the blood plasma is 101.5 milli-equivalents per kilo (Sjollema and Seekles, 1933), then it may be assumed that the 11.2 milli-equivalents of chloride in the 1 kilo of muscle (see Table II) are in solution in 110 cc. of water. If the total water in 1 kilo of muscle is 770 cc., then 660 cc. are fiber water, and the initial concentrations of potassium, magnesium, and creatine in the fibers are 180, 38, and 58 milli-equivalents per kilo, respectively. Failure to allow for amounts of these substances originally present in tissue spaces can have very little effect, since the potassium so located, for example, is only about 0.5 milli-equivalent per kilo. The fiber volumes of the dystrophic animals are calculated from the potassium, magnesium, and creatine contents on the assumption that the concentrations remain the same as in the normal fiber water. For chloride, the tissue spaces are calculated from the chloride content of the dystrophic muscles as already described for the normal muscle, the remaining water being assumed to belong to the fibers.

TABLE III

Calculated Amounts of Fiber Water in 1 Kilo of Muscle of Dystrophic Rabbits

Normal volume = 666 cc.

Basis for calculation	Diet 11	Grain diet
	cc.	cc.
K	440	374
Mg	445	475
Creatine	371	(545)
Chloride	377	416

The results (Table III) show uniformly that the fiber water has decreased from 666 cc. to 371 or 475 cc. It is particularly striking that the values calculated from the chloride gains are about equal to those calculated by very different assumptions from potassium, magnesium, and creatine. The high value of 545 cc. of fiber water calculated from the creatine content of the muscles of the animals on the grain diet may perhaps be discounted on the theory that this is somehow related to the high degree of calcification found in many of these muscles.

Theoretically, all the fiber volumes calculated in Table III for a given diet should be equal if the changes are due simply to a decrease in the number of intact fibers. Considering the experimental error, the agreement seems close enough to offer support for this theory as a first approximation. The changes which we are reporting, therefore, are approximately those which might have been predicted from the histology of the muscles, where evidence of the disintegration of some fibers is observed.

It is possible, however, that the results are due in part to an alteration in the composition of the intact muscle fibers rather than to a mere decrease in the number of fibers of normal composition. It might be suggested that evidence in favor of this view could be derived from the studies of Victor (1934) on similar dystrophic rabbit muscles. His report showed that the rate of oxygen consumption of the muscle was increased, as well as the rheobase and the chronaxie. The high metabolic rate might have been due, however, to a few disintegrating fibers, and the changes in the constants of electrical excitability may have indicated merely the prior disintegration of the most excitable of the fibers in the muscle.

As regards their electrolyte changes, the muscles which we have observed are similar to muscles of guinea pigs raised on a diet deficient in vitamin C (Randoin and Michaux, 1932) and similar also to denervated rabbit muscles, which show nearly equal percentage decreases in potassium creatine, and acid-soluble phosphate (Hines and Knowlton, 1933), as well as corresponding increases in chronaxie (Leulier, Pomme, and Richard, 1932).

SUMMARY

Analyses of normal and pathological rabbit muscles show that nutritional muscular dystrophy is associated with a gain of chloride, presumably proportional to the increase in interstitial fluid and a corresponding loss of potassium, magnesium, and creatine, presumably proportional to the loss in number of intact fibers.

High concentrations of calcium and total phosphorus were found

on analysis of muscles showing histological evidence of calcification.

BIBLIOGRAPHY

Fenn, W. O., *Physiol. Rev.*, **16**, 450 (1936).
Fenn, W. O., and Cobb, D. M., *Am. J. Physiol.*, **115**, 345 (1935).
Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
Goettsch, M., and Brown, E. F., *J. Biol. Chem.*, **97**, 549 (1932).
Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.*, **54**, 145 (1931).
Hines, H. M., and Knowlton, G. C., *Am. J. Physiol.*, **104**, 379 (1933).
Leulier, A., Pomme, B., and Richard, *Compt. rend. Acad.*, **194**, 1280 (1932).
Madsen, L. L., *J. Nutrition*, **11**, 471 (1936).
Madsen, L. L., McCay, C. M., and Maynard, L. A., *Cornell Univ. Agric. Exp. Stat., Memoir 178*, 3 (1935).
Morgulis, S., and Spencer, H. C., *J. Nutrition*, **11**, 573 (1936, *a*); **12**, 173 (1936, *b*).
Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, **15**, 283 (1913).
Pappenheimer, A. M., and Goettsch, M., *Proc. Soc. Exp. Biol. and Med.*, **34**, 522 (1936).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Randoin, L., and Michaux, A., *Compt. rend. Acad.*, **194**, 647 (1932).
Rose, W. C., Helmer, O. M., and Chanutin, A., *J. Biol. Chem.*, **75**, 543 (1927).
Salit, P. W., *J. Biol. Chem.*, **96**, 659 (1932).
Shohl, A. T., and Bennett, H. B., *J. Biol. Chem.*, **78**, 643 (1928).
Sjollema, B., and Seekles, L., *Biochem. Z.*, **264**, 316 (1933).
Sunderman, F. W., and Williams, P., *J. Biol. Chem.*, **92**, 99 (1931).
Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923-24).
Victor, J., *Am. J. Physiol.*, **108**, 229 (1934).
Wang, C. C., *J. Biol. Chem.*, **111**, 443 (1935).

A RAPID PHOTOELECTRIC METHOD FOR THE DETERMINATION OF GLUCOSE IN BLOOD AND URINE

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The reduction of potassium ferricyanide to ferrocyanide by glucose has been used as the basis of several successful quantitative methods for glucose. In the most popular of these, the Hagedorn and Jensen (1) method, the excess of ferricyanide is determined by iodometric titration. In the Folin (2) micromethod, the ferrocyanide formed is converted to Prussian blue and measured colorimetrically. Only in the Hawkins and Van Slyke (3) method is use made of the fact that ferricyanide solutions are yellow and ferrocyanide colorless. In this method, glucose is estimated by measuring the time required completely to decolorize a ferricyanide solution.

The Hawkins and Van Slyke procedure is easy and rapid. Its apparent lack of popularity is probably due to the fact that the exact end-point is difficult to determine and that any error made is irreparable. Furthermore, it does not lend itself to multiple determinations. The present author has been able to obviate the difficulties of the Hawkins and Van Slyke method and yet retain the principle involved. The resulting method is simple, easy, and accurate. In it, the diminution in the yellow color of an excess of ferricyanide by glucose is measured in a photoelectric colorimeter.

Method

Alkaline Ferricyanide Reagent—Solution A, 1.8000 gm. of $K_3Fe(CN)_6$, purified according to Peters and Van Slyke (4), and 30 gm. of anhydrous Na_2CO_3 are made up to a liter with distilled water. Solution B, 65 cc. of Solution A are made up to 100 cc. These solutions are kept in dark bottles away from sunlight.

Procedure

Folin-Wu Filtrates of Blood—To exactly 1 cc. of Folin-Wu filtrate in a small test-tube or centrifuge tube marked for 8 cc. are added exactly 2 cc. of the diluted alkaline ferricyanide reagent (Solution B). The tube is immersed in boiling water for 5 minutes, cooled under the tap, diluted to the mark, mixed by inversion, and read in the photoelectric colorimeter, water set at 100, and with a blue filter. (The author used the Cenco-Sheard-Sanford photelometer (5) with bakelite inserts for micro work.) If the solution has been completely decolorized, the determination is repeated with 0.5 cc. of filtrate and 0.5 cc. of water.

Finger Drop Blood—A dilute tungstic acid solution is made up by mixing 8 cc. of 10 per cent sodium tungstate and 8 cc. of $\frac{2}{3}$ N H_2SO_4 and diluting to 100 cc. To 2.5 cc. of dilute tungstic acid in a centrifuge tube is added 0.1 cc. of blood. If the pipette is graduated "to contain," it is washed out several times with the tungstic acid in the tube. After the mixture has been well stirred, the protein precipitate is centrifuged off. 2 cc. of the clear filtrate are transferred to another tube marked for 8 cc. and to it is added exactly 1 cc. of the alkaline ferricyanide reagent (Solution A). The tube is placed in boiling water for 5 minutes, cooled, diluted to 8 cc., mixed, and read in the photoelectric colorimeter.

Urine—To 10 cc. of urine are added 5 cc. of approximately 0.1 N oxalic acid and 5 cc. of water. The solution is mixed and then shaken for 2 minutes with about 1.5 gm. of Lloyd's reagent and filtered. 5 cc. of this filtrate are diluted in such a way as to give a solution containing from 0.1 to 0.2 mg. of glucose per cc. To 1 cc. in a tube marked for 8 cc. are added 2 cc. of Solution B and the reducing action is determined as in the case of the Folin-Wu filtrates. For less accurate work, dilutions of urine can be made without Lloyd's reagent.

Calculations are made by reference to a curve or table of microammeter readings prepared from known glucose solutions. Thus, if 10 cc. of a stock glucose solution containing 2 mg. of glucose per cc. are diluted to 100 cc., then determinations made with 0, 0.2, 0.5, 0.8, and 1.0 cc. respectively made up to 1.0 cc. will give values for glucose solutions equivalent to Folin-Wu filtrates from bloods containing 0, 40, 100, 160, and 200 mg. of glucose per 100 cc.

respectively. For finger drop blood, a similar curve is prepared by using glucose solutions in which 2 cc. represent 20/26 of the glucose in 0.1 cc. of blood. Curves B and C (Fig. 1) are two such curves plotted on semilogarithmic paper. For the sake of convenience, a third curve may be prepared (Curve A) with 4 cc. of Solution B and 2 cc. of Folin-Wu filtrate. With such a curve, determinations of glucose in bloods of concentrations higher than 200 mg. per 100 cc. can be saved by adding another 2 cc. of ferri-

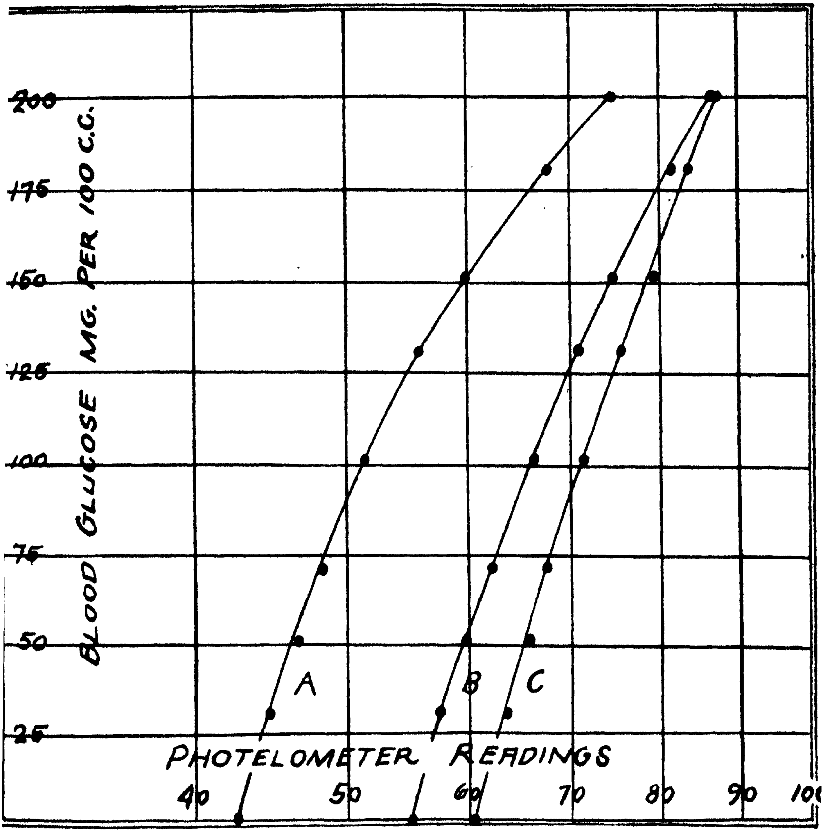

FIG. 1. The relationship between photelometer readings and concentration of glucose, as plotted on semilogarithmic paper. Curves A and B are for 2 and 1 cc. of Folin-Wu filtrates respectively. Curve C is for finger drop blood filtrates.

cyanide to the tube which has been found to be decolorized and 1 cc. of water, heating for 3 more minutes, and reading the value on Curve A instead of Curve B and multiplying by 2, since only 1 cc. of Folin-Wu filtrate had been used.

The advantages of this method for glucose are obvious. Only one reagent is necessary. This reagent is inexpensive, easy to make up, and has been found to be stable for at least 2 months. The method is simple and rapid. It requires only 7 minutes to make the determination on a blood or urine filtrate. If a blank determination is run simultaneously to make sure that the ferricyanide solution has not deteriorated, it is almost impossible to make an error in the determination.

TABLE I

Accuracy of Determination of Glucose

The results are expressed in terms of mg. of glucose per 100 cc.

Sample	Method		Added glucose	Total glucose	
	Folin phosphomolybdate (6)	Hoffman		Calculated	Found
Normal blood	99	102			
" "	105	104			
" "	108	109	100	209	212
" "	104	106	50	156	155
" "	92	94	80	174	176
Diabetic "	168	166	200	366	360
" "	232	230			
" "	285	290			
Normal urine	210	270	2000	2270	2230
" " (with Lloyd's reagent)	100	120	1000	1120	1110
Diabetic " " " "	5240	5320			
" " " " "	1600	1620	1000	2620	2640

Table I shows that the values for glucose obtained with blood and urine are comparable with those obtained with the Folin phosphomolybdate method. Added glucose is accurately recovered. Normal postabsorptive glucose concentrations in blood by this method in sixteen normal persons ranged between 90 and 105 mg. per 100 cc.

SUMMARY

A method for the determination of glucose in blood and urine is offered which depends upon the photoelectric measurement of the diminution of color in a ferricyanide solution on reduction with glucose.

The method is simple, rapid, and reliable.

BIBLIOGRAPHY

1. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).
2. Folin, O., *J. Biol. Chem.*, **77**, 421 (1928).
3. Hawkins, J. A., and Van Slyke, D. D., *J. Biol. Chem.*, **81**, 459 (1929).
4. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 462 (1932).
5. Sanford, A. H., Sheard, C., and Osterberg, A. E., *Am. J. Clin. Path.*, **3**, 405 (1933).
6. Folin, O., *J. Biol. Chem.*, **82**, 92 (1929).

THE PHOTOELECTRIC DETERMINATION OF POTASSIUM IN MINUTE QUANTITIES OF SERUM

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In the Jacobs and Hoffman (1) procedure for the determination of potassium in serum, potassium is precipitated as $K_2NaCo(NO_2)_6$ as in the Kramer and Tisdall (2) method, the washed precipitate is decomposed with boiling water, and the cobalt is determined by measuring the emerald-green color formed on the addition of choline hydrochloride and sodium ferrocyanide.

This colorimetric method can be easily adapted to the Cenco-Sheard-Sanford photelometer (3). The green solutions when measured in the photoelectric colorimeter with a blue filter give values on the microammeter, with water set at 100, which, when plotted on semilogarithmic paper, give smooth curves (see Fig. 1). Curve A is obtained when 1 cc. of serum is used; that is, the value for 0.2 mg. of K is calculated as 20 mg. of K per 100 cc. Curves B and C represent values with 0.5 cc. and 0.2 cc. of serum respectively. Curve C is sufficiently near to a straight line to be expressible in terms of the linear equation $C = 200 \log 98.5/R$, where C is the concentration in mg. of K per 100 cc. and R is the corresponding reading on the microammeter. This curve has sufficient slope to make the determination of potassium in 0.2 cc. of serum a practical procedure.

Several modifications of the original Kramer-Tisdall and Jacobs-Hoffman procedures have been found desirable. The precipitation technique has been modified to permit determinations directly on old serum as well as fresh serum. The addition of sodium acetate was found to prevent the precipitation of protein and extra cobaltinitrite, apparently by adjusting the pH more to the alkaline side of the isoelectric point of the serum proteins. Furthermore, a

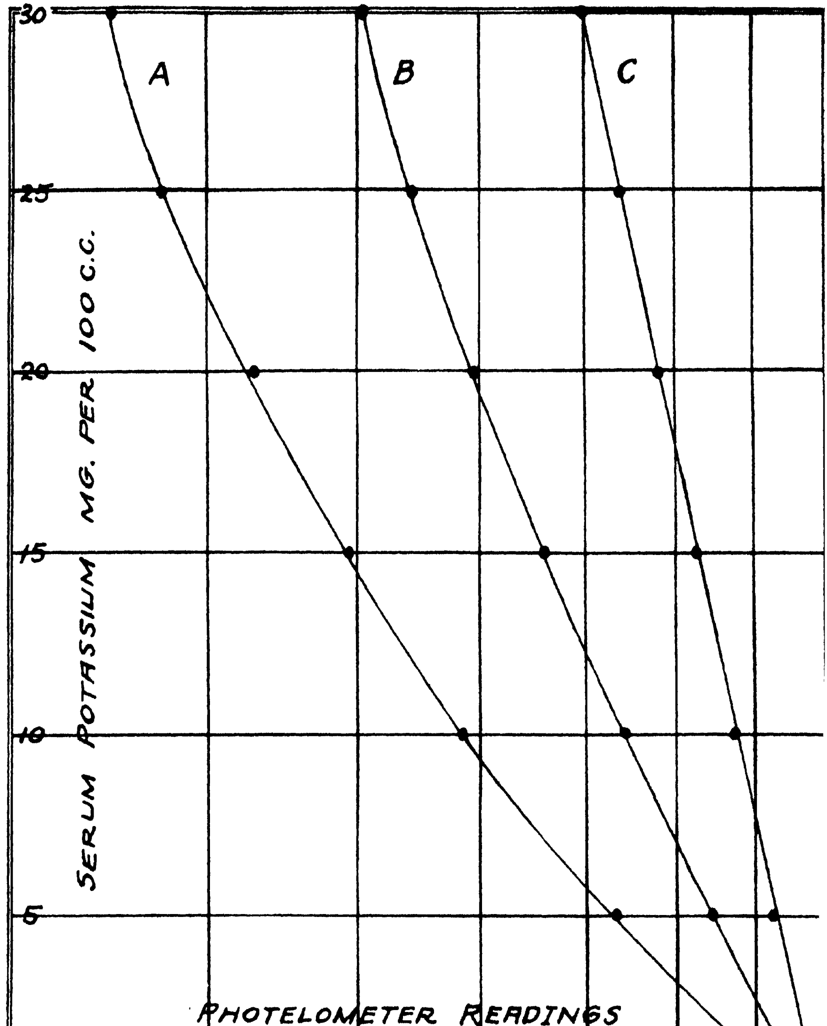

FIG. 1. Curves showing the relationship between photelometer readings and concentration of potassium. Curve A represents determinations made with 1 cc. of serum; Curves B and C, 0.5 and 0.2 cc. of serum respectively.

new wash fluid has been prepared for the first washing, which removes practically all precipitated or dissolved protein and thus permits the subsequent washings to be made with 70 per cent alcohol without danger of further precipitation of protein.

It was found necessary to modify the ferrocyanide reagent. Since photoelectric measurements depend upon the permanency of the curves obtained, the reagents used must be stable and give constant blanks. Dilute solutions of the colorless sodium ferrocyanide, however, are rapidly oxidized spontaneously to the yellow ferricyanide. This change can be avoided by using a saturated solution of the more soluble potassium ferrocyanide in oxygen-free water, and making up the dilute solution only at the time of the determination.

Method

Reagents—

0.2 per cent choline hydrochloride in water.

Saturated potassium ferrocyanide in previously boiled water. A small quantity is prepared and kept in a dark bottle in the refrigerator. It will keep for at least 2 months. At the time of the determination, 0.2 cc. of this solution is made up to 25 cc.

Saturated sodium acetate solution in water.

Sodium cobaltinitrite reagent. Solution A, 25 gm. of cobaltous nitrate are dissolved in 50 cc. of water and to this solution are added 12.5 cc. of glacial acetic acid. Solution B, 120 gm. of sodium nitrite are dissolved in 180 cc. of water. To all of Solution A are added 210 cc. of Solution B. An evolution of gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent is kept in the refrigerator and is filtered each time before using.

Wash Solution A. 10 per cent ethyl alcohol is saturated by shaking with pure, freshly precipitated $K_2NaCo(NO_2)_6$. Some of it is filtered just before using.

70 per cent ethyl alcohol.

Procedure

To 0.2 cc. of serum in an ordinary 15 cc. conical centrifuge tube marked for 8 cc. are added 0.1 cc. of saturated sodium acetate and 0.3 cc. of sodium cobaltinitrite reagent. The latter is added in two portions, and mixed well after each addition. The contents are allowed to stand for 45 minutes Then the walls of the tube are washed down with 0.5 cc. of water. The tube is centrifuged for 15 minutes and drained by inversion for at least 2 minutes. The precipitate is then thoroughly stirred with about 3 cc. of Wash Solution A, centrifuged again for 10 minutes, and the tube inverted and drained. The precipitate is now washed twice with about 2 cc. of 70 per cent alcohol and drained thoroughly after each centrifugation.

If the determination is to be made on ashed material or other inorganic solutions, 0.2 cc. of the solution containing about 0.04 mg. of K is diluted with 0.2 cc. of the saturated sodium acetate and treated with 0.2 cc. of the sodium cobaltinitrite reagent. The tube is washed down with 0.5 cc. of water after 45 minutes, centrifuged, drained, and washed twice with 70 per cent alcohol; the washing with Wash Solution A is omitted.

TABLE I

Accuracy of Determination of Potassium

The results are expressed in terms of mg. of potassium per 100 cc.

Sample No.	Hoffman method		Jacobs-Hoffman method
	Serum	Ash	Ash
1	19.4	19.0	19.2
2	22.4	22.3	22.0
3	17.6	18.0	18.2
4	16.0	16.4	16.3
5	20.1	20.4	20.1

The washed precipitate is dissolved by covering with about 3 cc. of water and placing in a boiling water bath for about 10 minutes. To make sure that none of the precipitate has escaped above the surface of the liquid, the walls of the tube are scoured with a stirring rod and the tube replaced for several minutes in the water bath. The tube is now cooled to room temperature. 1 cc. of choline hydrochloride is added, followed by 1 cc. of the dilute ferrocyanide, and then by water to the 8 cc. mark. The contents are mixed by inversion, and read in the photoelectric colorimeter, water set at 100, and with a blue filter. Simultaneously, a blank containing only water, choline, and ferrocyanide is made up and read in the colorimeter.

The concentrations can be read off directly from Curve C or can be calculated from the equation of the curve. If the blank has altered considerably, a fresh ferrocyanide reagent must be prepared. If, however, the change is only slight, the reading of the unknown can be corrected by multiplying it by the ratio of the original blank to the new blank. This correction is possible because the new blank will give a new curve parallel to the original curve with the same constant in the equation.

In preparation of the curves, a series of determinations was made on 0.04 mg. of K in 0.2 cc. of solution. The average of these readings was determined, and a cobaltous nitrate solution prepared, 0.2 cc. of which gave the same reading. The curves were then made from the variations in the concentration of this known cobalt solution.

Table I shows the accuracy of the method by comparing the determinations made by the micromethod on serum with those on ashed serum and with those by the Jacobs-Hoffman macromethod. The standard deviation from the mean on twenty determinations of the same serum was ±2.8 per cent.

SUMMARY

The Jacobs-Hoffman colorimetric method for serum potassium has been modified and adapted to the photoelectric colorimeter. Determinations can be made on 0.2 cc. of serum with an accuracy of 3 per cent.

BIBLIOGRAPHY

1. Jacobs, H. R. D., and Hoffman, W. S., *J. Biol. Chem.*, **93**, 685 (1931).
2. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, **46**, 339 (1921).
3. Sanford, A. H., Sheard, C., and Osterberg, A. E., *Am. J. Clin. Path.*, **3**, 405 (1933).

OSMOTIC PRESSURE, MOLECULAR WEIGHT, AND STABILITY OF AMANDIN AND EXCELSIN AND CERTAIN OTHER PROTEINS

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The present work[1] is a continuation of previous studies (1, 2) of the osmotic pressures of proteins in mixed solvents, especially in concentrated aqueous solutions of urea. In this earlier work it was found that the molecular weights of edestin and hemoglobin, in urea solution, were only one-fourth or one-half as great as in aqueous solutions. Egg albumin and serum albumin, on the other hand, were found to possess stability with respect to their molecular weights, which were not changed by the presence of urea. All of the proteins studied were denatured by urea. Their stability or instability, therefore, shows no general correlation to their denaturation. In this work other factors are considered.

Hopkins (3) has shown the presence of sulfhydryl groups in egg albumin after treatment with urea. This is also true of edestin. The appearance of thiol groups after denaturation by agents other than urea has been found by Mirsky and Anson (4) to occur in the case of these and other proteins. On the other hand, a large number of proteins (serum albumin, serum globulin, gliadin, and zein, for example), although rich in cystine, show no detectable sulfhydryl groups after solution in urea or denaturation by other means. Goddard and Michaelis (5) have recently studied a sulfhydryl protein derived from wool and their findings have been useful in the elucidation of the structure of the keratins. Cohn (6), from a comparison of the molecular weights of certain proteins in aqueous and urea solutions, has suggested the presence of a cyclic structure of cystine in proteins. That the splitting of proteins into smaller units of definite size may occur in the human body has been intimated by Calvery and Freyberg (7).

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[1] The experimental work was done in 1931-33. The writer is indebted to Dr. David I. Hitchcock for revising the manuscript.

It seemed of importance, therefore, to obtain additional data in regard to possible correlations between protein instability and sulfur linkage activity. For this purpose osmotic pressure measurements on two more representative proteins have been carried out in aqueous and urea solutions, and accompanying tests made for the presence or absence of —SH groups. Amandin and excelsin, two oil-seed globulins, of widely differing cystine content, were chosen for this purpose because of their similarity to edestin and because they show this characteristic property of spontaneously becoming sulfhydryl proteins when dissolved in urea solution.

EXPERIMENTAL

Excelsin was prepared in the following way (*cf.* (8)). Brazil nuts were shelled and the kernels (without removal of their skins) chilled to about 1° and then ground in a meat grinder with a perforated disk type of cutter. The ground kernels were placed in a folded cotton cloth and the oil expressed in a hydraulic press. After the material was reground, 400 gm. of the meal were suspended in 2 liters of 3 per cent ammonium sulfate solution, and the mixture was shaken gently at room temperature for 24 hours and then filtered. To the filtrate, ammonium sulfate was added to the extent of 60 per cent saturation. After the resulting mixture had stood at 1° for 4 hours, the precipitate which formed was filtered off and dissolved in 6 per cent ammonium sulfate. After filtration, the precipitation by ammonium sulfate at 60 per cent saturation was repeated. The material was then washed with ammonium sulfate solution (60 per cent saturated), dissolved in water, and dialyzed in collodion sacks at room temperature, with stirring, against distilled water (saturated with toluene), until the outer fluid showed no test for sulfate. The excelsin, which precipitated partly in crystalline form, was dissolved in 6.66 M urea, 0.05 M with respect to acetate or phosphate buffer, also in 50 per cent glycerol, 0.2 M with respect to phosphate buffer, at a pH approximately that of the isoelectric point. It was observed that after the stock urea solution of excelsin had stood for some time a slight cloud of precipitate formed which resisted filtration from this concentrated solution (about 6 per cent). Filtration, after dilution of the stock solution 6 times with urea solution, brought about its complete removal. The filtrate was brought back to the original concentration of protein by ultrafiltration. The insoluble material, although insignificant in quantity, was probably of a lipid nature, since it rose toward the surface of the solution.

Amandin was prepared as follows (*cf.* (8)). Almonds were soaked in cold water for several hours and their skins removed. The meats, after being chilled to about 1°, were ground in a meat grinder; the meal so obtained was placed in a folded canvas cloth and the greater part of its oil was removed by means of a hydraulic press; the remaining oil and fatty material were removed by an extraction with aviation gasoline. After the material was dried in air, 400 gm. were suspended in 2 liters of 10 per cent NaCl solution and the mixture was gently shaken overnight at room temperature. After the undissolved material had settled, the solution was siphoned off, and the residue was treated in the same way with 1 liter of 10 per cent NaCl. The extraction was repeated once more, with 500 cc. of sodium chloride solution. To the combined extracts was added an equal volume of saturated ammonium sulfate solution, and the resulting mixture was allowed to stand for 4 hours in a cold room at 1°. The suspension of the precipitate, which remained after the solution was siphoned off, was centrifuged, the supernatant liquid discarded, and the material dissolved in water. The solutions were centrifuged to remove any insoluble material, and then dialyzed against distilled water (saturated with toluene) at room temperature until the outer solutions showed no test for sulfate. The amandin which was precipitated in the dialysis was used to prepare the solutions of the composition recorded in the osmotic experiments.

Protein concentration was determined colorimetrically with the phenol reagent of Folin and Ciocalteu (9), by a method like that of Greenberg (10). Protein standards were used in the colorimetric comparison. The standard solution of excelsin was prepared as follows: A portion of the excelsin, precipitated in dialysis, was washed four times with distilled water by decantation, and then just dissolved by careful addition of 0.05 N NaOH. The protein content of the alkaline solution (pH about 8), after it had been brought to known volume, was determined by drying a sample to constant weight at 105°, and correcting the weight of the residue for the sodium present. The standard solution was an aliquot portion of the basic solution, neutralized and made to correspond in composition to the solvents used in the osmotic experiments; *i.e.*, 6.66 M urea, 0.05 M with respect to buffer at pH 6.3; and 50 per cent glycerol, 0.2 M with respect to buffer at pH 5.5. A second standard was prepared from dry excelsin by dissolving 1 gm. of material in 100 cc. of urea-buffer solution at pH 6.3. The dry excelsin was prepared by washing the precipitated excelsin by decantation, first with distilled water, then with gradually increasing concentrations of alcohol, followed by absolute alcohol and finally with absolute ether. After the preparation was dried in air at room temperature, its water content was determined and the corresponding correction applied to the standard. The two standards agreed in the colorimetric analysis to less than 1 per cent. Standard solutions of amandin were prepared by the first procedure given above for excelsin.

The osmotic pressure measurements were carried out upon solutions of amandin and excelsin contained in collodion sacks which were fitted to glass manometer tubes to form a pendulum type of osmometer (1). At 25° the solutions were stirred by moving the osmometers back and forth in a thermostat by means of a Warburg apparatus. At 0°, the solutions were stirred outside the temperature bath in a cold room at about 1° until the manometer heights became approximately constant, and then they were placed in a bath of melting ice until the pressure became constant. This required from 1 to 2 weeks at 0°, but at 25° a shorter time sufficed. When the manometer heights, which were recorded daily, became constant, the osmometers were taken apart and the inner solutions analyzed for protein and the outer solutions qualitatively tested for protein with tannic acid. In most experiments the tests were completely negative, showing that the membranes used were strictly semipermeable and that protein products of low molecular weight were not appreciably present in the solutions. In cases in which protein was found, the amount was small, less than 1 part in 10,000, as roughly estimated by comparison of turbidities against solutions of known protein content. This error was assumed to be negligible. The measurements were corrected for capillary rise, which was determined in a 1 per cent solution of protein at the temperature of the experiment.

Buffer solutions were prepared according to the data of Cohn (11). For the preparation of urea solutions of known pH, the dissociation-titration curves of these buffers in 6.66 M urea, as previously reported (1) and slightly revised, were employed. In most cases, however, the reported pH values were obtained from electrometric measurements with a hydrogen or quinhydrone electrode at the end of an osmotic experiment, a saturated KCl junction and 0.1 N KCl (or saturated) calomel cells which were checked by means of Michaelis' standard acetate buffer (12) being used. The temperature was controlled by thermostat regulation at 25° or 30° and the pH values were calculated according to Clark (13), the standard being Sörensen's original value of 0.3380 volt for the 0.1 N calomel cell at 18°.

Other details of experimental procedure not given here will be found in a previous paper (2).

Osmotic Pressure and Molecular Weight of Native Amandin and Excelsin

Amandin—Two osmotic pressure measurements were carried out upon amandin in 0.2 M acetate buffer solution at pH 5.4, which is close to the isoelectric point of this protein (14). The results are given in Table I. The molecular weight of the protein, given in Table I, was calculated from these measurements by means of the van't Hoff-Morse equation, which is

$$M = \frac{C}{P} \times RT \qquad (1)$$

where

M = molecular weight in gm. dry protein
P = osmotic pressure in cm. H_2O of density 1
C = concentration of dry protein in gm. per 100 cc. solvent
RT = gas constant × absolute temperature, $0.08207 \times 273.1 \times 76 \times 13.596 \times 10 = 2.315 \times 10^5$ (100 cc. × cm. H_2O per gm. molecule) at 0°, or 2.528×10^5 at 25°

The mean value obtained for the molecular weight of native amandin from these measurements is 206,000. This value is not corrected for deviation from the ideal solution law, which, for substances of high molecular weight, is expressed by Equation 1. The extent of the deviations from this equation was not obtained owing to the limited solubility of amandin in 0.2 M buffer solution at pH 5.4, which prevented measurements at a concentration greater than 1.87 gm. per 100 cc. of solvent. Measurements in concentrated salt solution in which the protein is more soluble are open to objections (*cf.* Adair and Callow (15)). There is, however, reason for believing that the measurements are represented fairly closely by Equation 1. Adair and Robinson (16), in discussing their osmotic pressure measurements of serum globulin in similar solutions (0.2 M phosphate buffer at pH 5.35), state, "In dilute solutions, containing less than 4% of protein, the pressure is practically proportional to the protein concentration if the reaction is not too far from the isoelectric point."

TABLE I

Molecular Weight of Amandin from Measurements of Its Osmotic Pressure in Aqueous Buffer Solution

Solvent, 0.2 M acetate buffer; pH 5.4; temperature 25°; time of equilibrium, 3 to 4 days.

Experiment No.	C Concentration per 100 cc. solvent	P Osmotic pressure	$\frac{P}{C}$	M Molecular weight
	gm.	*cm. H_2O*		*gm.*
201	1.84	2.33	1.27	199,000
202	1.87	2.22	1.19	212,000
Mean				206,000

The above value for the molecular weight of amandin obtained from osmotic pressure experiments agrees well with that obtained by Svedberg and Sjögren (8) from ultracentrifugal measurements, namely 208,000 ± 5000.

Excelsin—Excelsin, like amandin, dissolves but slightly in dilute buffer solutions in the neighborhood of its isoelectric point. In order to increase the solubility, glycerol was employed to the extent of a 50 per cent solution. 50 per cent glycerol did not appear to denature excelsin, and solutions of this protein in it remained sterile.

Measurements of the osmotic pressure of solutions of increasing excelsin concentration, made with buffer adjusted to the isoelectric point of the protein (taken as pH 5.5 (8, 14)), are given in Table II. From Table II it is seen that the osmotic pressure is not proportional to the protein concentration, but increases less than corresponds to the increase of protein concentration. The solutions are, therefore, not ideal and the measurements were extrapolated to infinite dilution, where the ideal solution law holds, by plotting C/P against C, and drawing the best straight line through the points (Fig. 2). The intercept on the ordinate axis gives a value of $C/P = 0.85$, or $P = 1.18$ cm. of water per gm. of protein in 100 cc. of solvent, which corresponds to the osmotic pressure of an ideal solution of excelsin. In order to obtain C/P values which conform to the ideal solution law at the measured concentrations, use was made of the following equations

TABLE II

Molecular Weight of Excelsin from Measurements of Its Osmotic Pressure in Glycerol Solutions

Solvent, 50 per cent* glycerol; 0.2 M in phosphate buffer at pH 5.5; temperature 25°.

Experiment No. (1)	C Concentration per 100 cc. solvent (2)	P Osmotic pressure (3)	g† Osmotic coefficient (4)	C_0‡ Corrected concentration (5)	$\frac{P}{C_0}$ (6)	M Molecular weight (7)
	gm.	*cm. H_2O*				*gm.*
2	1.97	2.24	0.945	1.86	1.20	211,000
3	3.49	3.69	0.912	3.18	1.16	218,000
4	6.43	6.42	0.845	5.43	1.18	214,000
Mean						214,000

* Per cent by volume.

† Calculated from the equation, $g = C_0/C$.

‡ Calculated from the equation, $C_0 = C + BPC$, where, from Fig. 2, $B = -0.0242$.

which have been previously shown (2) to be applicable to measurements which fall on a straight line when C/P is plotted against C:

$$C_0 = C + BPC \qquad (2)$$

$$\frac{C_0}{P} = \frac{C}{P} + BC \qquad (2, a)$$

where

C_0 = corrected concentration of protein
$-B$ = a constant, equal to the slope of the line in the plot of C/P *versus* C
$\frac{C_0}{P}$ = ratio of concentration of protein to the osmotic pressure which conforms to the ideal solution law

From the ideal solution law ratios so obtained (Column 6 of Table II), values of M, calculated at each protein concentration by means of Equation 1, are given in Column 7 of Table II. The mean value for the molecular weight of native excelsin is 214,000. This value agrees well with that obtained by Svedberg and Sjögren from measurement in the ultracentrifuge upon aqueous salt solutions of excelsin at the isoelectric point; namely, 212,000.

Molecular Weight of Amandin and Excelsin in Urea Solution

Excelsin—In the estimation of the molecular weight of a protein from osmotic pressure measurements, it is expedient that such measurements be made in solutions at the isoelectric point of the protein, or very close to it, in order to avoid the complicating effects of a Donnan membrane equilibrium (1, 2, 17). Since the isoelectric point of a protein in urea solution is appreciably different from that in aqueous solution, this constant for excelsin in 6.66 M urea was first determined osmotically;and found to extend over the range pH 6.3 to 6.75 (Fig. 1).

Measurements of the osmotic pressure of solutions of excelsin of increasing protein concentration in urea solution at pH 6.3 are given in Table III. From Table III it is seen that the pressure is not proportional to the protein concentration, which is expressed in gm. per 100 cc. of solvent, but increases slightly more than is proportional to the increase in the concentration. A plot of C/P against C (Fig. 2) gave points which fell fairly well on a straight line. Hence Equation 2 is applicable to the measurements, and the data were then corrected so as to conform to the ideal solu-

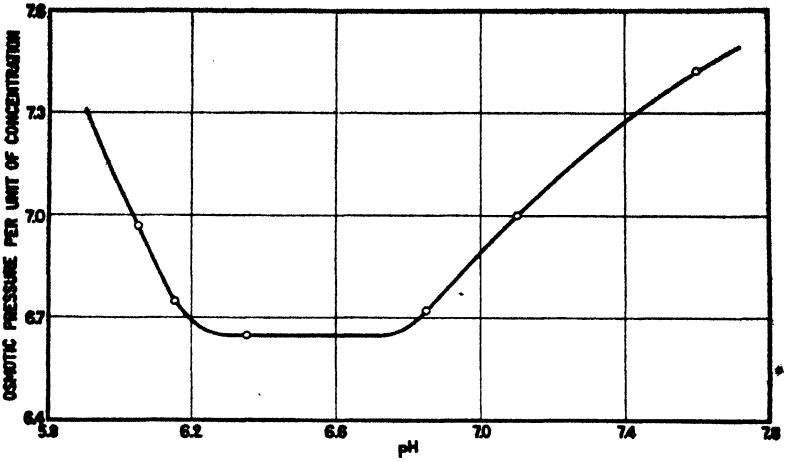

Fig. 1. Influence of the hydrogen ion activity on the osmotic pressure of excelsin. Location of the hydrogen ion activity at which the osmotic pressure is a minimum, pH 6.3 to 6.75. Solvent, 6.66 M urea, 0.05 M in phosphate buffer. Protein concentration, 1.12 to 1.18 gm. per 100 cc. of solvent.

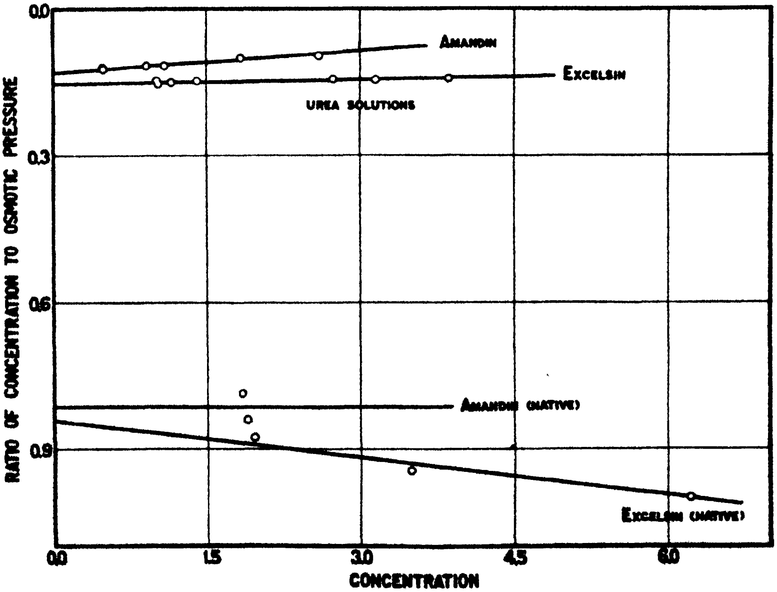

Fig. 2. Comparison of the osmotic pressures of amandin and excelsin in urea solutions with those in aqueous buffer solutions (amandin, native) and in glycerol solutions (excelsin, native).

tion law. The P/C_0 values so obtained (Column 6 of Table III) showed no trend with concentration.

The mean value obtained for the molecular weight of excelsin in urea solution, calculated by means of Equation 1, from pressure measurements which are at a minimum with respect to the effect of hydrogen ion activity and which are corrected for deviation, is 35,700 (Column 7 of Table III).

TABLE III

Molecular Weight of Excelsin from Measurements of Its Osmotic Pressure in Urea Solution

Solvent, 6.66 M urea; 0.05 M in acetate buffer; pH 6.3; temperature 0°; time of protein in urea at end of experiments, 15 days.

Experiment No. (1)	C Concentration per 100 cc. solvent (2)	P Osmotic pressure (3)	g* Osmotic coefficient (4)	C_0† Corrected concentration (5)	$\frac{P}{C_0}$ (6)	M Molecular weight (7)
	gm.	*cm. H_2O*				*gm.*
517	1.02	6.99	1.03	1.05	6.66	34,800
513	1.04	6.81	1.03	1.07	6.36	36,400
510‡	1.16	7.71	1.03	1.19	6.48	35,700
514	1.42	9.55	1.04	1.47	6.50	35,600
511	2.73	18.90	1.07	2.91	6.49	35,700
512	3.15	22.01	1.08	3.40	6.47	35,800
516	3.87	27.40	1.10	4.25	6.45	35,900
Mean						35.700

* Calculated from the equation, $g = C_0/C$.

† Calculated from the equation, $C_0 = C + BPC$, where, from Fig. 2, B +0.00357.

‡ 0.05 M phosphate buffer was used in this experiment.

This figure is, within experimental error, one-sixth of the normal molecular weight. It is interesting to note that Svedberg and Sjögren (8) observed in the ultracentrifuge indications of such a splitting by means of alkali; in aqueous salt solutions at a pH of 11.88, only molecules having a sedimentation constant of the same order of magnitude as egg albumin (molecular weight, 34,500) were found. The fact that Svedberg and Sjögren observed no other molecules, representing intermediate stages in the breakdown, indicates that very probably the urea solutions are homomolecular, and not composed of a mixture of molecules of different weight whose average is 35,700.

Amandin—The osmotic pressures of solutions of increasing concentrations of amandin in 6.66 M urea were measured at pH 6.15, 0.15 M acetate buffer being used to maintain this reaction. The data are shown plotted in Fig. 2. The straight line for amandin in urea solution falls fairly close to that of excelsin in urea solution, but has a greater slope (B, for amandin, +0.0145; B, for excelsin, +0.00357), showing greater deviation from the ideal solution law for which $B = 0$.

TABLE IV

Molecular Weight of Amandin from Measurements of Its Osmotic Pressure in Urea Solution

Solvent, 6.66 M urea; 0.15 M in phosphate buffer; pH 6.15; time of protein in urea solution, 11 days.

Experiment No.	C Concentration per 100 cc. solvent	P Osmotic pressure	g* Osmotic coefficient	C_0† Corrected concentration	$\frac{P}{C_0}$	M Molecular weight
	gm.	*cm.* H_2O				*gm.*
1000	0.485	4.00	1.06	0.513	7.79	29,700
1007‡	0.487	3.92	1.06	0.515	7.61	30,400
1010‡	0.494	4.00	1.06	0.523	7.64	30,300
1008‡	0.920	7.84	1.11	1.02	7.69	30,100
1001	0.920	7.85	1.11	1.02	7.70	30,100
1002	1.09	9.35	1.14	1.24	7.54	30,700
1003	1.83	18.20	1.26	2.31	7.87	29,400
1004	2.60	26.90	1.39	3.65	7.37	31,400
Mean						30,300

* Calculated from the equation, $g = C_0/C$.

† Calculated from the equation, $C_0 = C + BPC$, where, from Fig. 2, $B = +0.0145$.

‡Time of protein in urea solution in these experiments, 22 days.

The mean value obtained for the molecular weight of amandin in urea solution, after correction of the data for deviation, is 30,300[2] (Table IV). Svedberg and Sjögren found aqueous salt solutions of amandin at pH 12.12 to contain two different centrifugible substances, namely normal amandin of molecular weight 208,000 and a dissociation product which was estimated to be about one-sixth of the normal molecule. In urea solution, this type of breakdown appears to be complete and uncomplicated by secondary reactions, since no change of molecular weight with time of the protein in urea solution was observed (Table IV).

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[2] This value may be subject to correction because it was obtained from solutions of pH 6.15, a value which was found to correspond with minimal solubility of amandin in urea solutions after 10-fold dilution with water.

Influence of Urea on Proteins

In addition to reduction of molecular weight, two other changes in amandin and excelsin take place in urea solution. The most obvious of these is the change of solubility. If the more concentrated solutions of amandin or excelsin in 6.66 M urea at a pH in the isoelectric region were diluted with water, after equilibrium in the osmometers had been reached, immediate precipitation of the protein occurred. The precipitated protein was insoluble in salt solution. Since denaturation has been defined as that change in proteins which occurs in solution, whereby the protein is rendered insoluble in solvents in which it was originally soluble, amandin and excelsin can be said to be denatured by urea.

The third change is a change in reactivity towards certain reagents; namely, those which react with —SH groups, as sodium nitroprusside or phosphotungstic acid. Amandin and excelsin, like egg albumin, produce no appreciable color with these reagents in aqueous solution, but do so after solution in 6.66 M urea. In accordance with the work of others (3, 18), the development of a magenta-pink color after the direct addition of sodium nitroprusside (and dilute ammonia), or a blue color with phosphotungstic acid (19, 20) (and phosphate buffer at pH 7 to 9, or sodium carbonate) in the solutions under consideration, is taken to indicate the presence of —SH groups in the protein.

The fact that there is a splitting of amandin and excelsin into smaller units, which is accompanied by formation of active —SH groups in the protein, indicates the possibility of a relationship

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If the isoelectric region for amandin in the concentrated solutions is the same as for excelsin (Fig. 1), it is estimated that the true molecular weight may be as high as 36,000 instead of 30,000. Owing to an unavoidable termination of the work, it has not yet been possible to decide this point by experiment.

between these two changes. This possibility would be strengthened if these two changes occurred as a general phenomenon in a great many proteins.

Some eight other proteins whose molecular weights have been determined in aqueous and urea solutions were therefore subjected to the sulfhydryl test. The results are given in Table V, from which it is seen that, in the case of amandin, excelsin, edestin, hemoglobin, and myogen in isoelectric urea solution, there is formation of —SH groups and reduction in molecular weight. In the case of serum albumin, serum globulin, gliadin, and pepsin, in isoelectric urea solutions, there is no dissociation, —SH groups do not form, and the molecular weights do not change. Egg albumin appears to be an exception to this relationship. This protein gives tests for the sulfhydryl group in urea solution, but its molecular weight is not detectably changed by this solvent. Thus, while there is a certain degree of correlation between the stability of a protein and the activity of the linkages of the sulfur atoms, explanation of the individual differences between the proteins in regard to size, reduction, or thiol formation, in view of the fact that all contain cystine, is not apparent.

We find that the results given in Table V may be explained and correlated by means of the following working hypothesis, in which a number of reasonable assumptions are made. These assumptions are:

1. That the splitting of proteins in isoelectric urea solution results from the rupture of the disulfide bonds of cystine (or other dithioamino acid).

2. That cystine, since it is a diaminodicarboxylic acid, exists in proteins in two types of structure, a cyclic structure, as suggested by Cohn (6), and a straight chain form corresponding to that in the amino acid itself. While there is no evidence for a cyclic structure of cystine alone, it seems possible that in some proteins it may be so linked as to form a ring, closed by other constituent parts of the protein molecule. Hence, for the simplest case of a protein containing 1 molecule of cystine in cyclic structure, we shall represent, in a gross way, its structure by the form of the letter A, in which the cross-bar represents the disulfide bond of cystine and the remaining part a long peptide chain. It is obvious that the rupture of only the S—S bond of cystine in this

TABLE V

Relation between Molecular Weight Changes and Appearance of —SH Groups in Certain Proteins at the Isoelectric Point

Protein (1)	Test* for —SH in aqueous solution (2)	Test† for —SH in urea solution (3)	Molecular weight in aqueous solution, Ma (4)	Molecular weight in urea solution, Mu (5)	Ratio $\frac{Mu}{Ma}$ (6)	Cystine content (7)	Moles‡ cystine per mole protein in urea (8)
						per cent	
Group I							
Egg albumin	—	+	35,000 (17)	36,000 (1)	1	1.22 (20)	2
Group II							
Hemoglobin	—§	+§	68,000 (21)	34,000 (1)	½	0.41 (22)	0.5
Myogen¶	**	+††	81,000 (23)	34,000 (23)	½		
Edestin	—	+	212,000 (25)	49,000 (1)	¼	1.36 (20)	3
Excelsin	—	+	212,000 (8)	36,000	⅙	1.84 (26)	3
Amandin	—	+	208,000 (8)	30,000	⅙ ⅐‡‡	0.85 (26)	1
Group III							
Serum albumin.	—	—	75,000 (2)	73,000 (2)	1	6.04 (20)	18
" globulin	—	—	175,000 (16)	174,000 (27)	1	2.2 (4)	16
Gliadin	—	—	42,000 (27)§§	44,000 (27)‖‖	1	2.18 (27)	4
Zein	—	—	34,000 (27)§§	33,000 (6, 27)	1	0.91 (22)	1
Pepsin	—	—¶¶	36,000 (28)	36,000***	1		

* Refers to tests upon isoelectric solutions

† Tests made within a few minutes or hours after the protein was dissolved in urea solution.

‡ Refers to the nearest integer (or reciprocal of an integer)

§ The natural color of hemoglobin interferes with the qualitative test. The tests as given are reported on the basis of the quantitative procedures of Mirsky and Anson (4), who employed acid acetone as their denaturing agent. It is assumed that this produced the same effect as urea with regard to —SH groups, since this is known to be true for egg albumin and other proteins

|| Mirsky and Anson (4) have obtained a higher value for cystine in hemoglobin; this would raise the figure in Column 8.

¶ This refers to the water-soluble protein of muscle. Weber and Stöver (23) have reported that urea reduced the molecular weight of myosin (muscle globulin). A sample of purified myosin, kindly supplied by Dr. J. T. Edsall, gave a positive test for —SH in isoelectric urea solution.

** Not reported for isoelectric solutions (pH 6.3). Mirsky (24) found some —SH groups in myogen at alkaline reactions, but the number increased with pH, indicating that they may be produced in native myogen by alkali.

†† Protein denatured by trichloroacetic acid (see Mirsky (24)).

‡‡ See foot-note 2.

§§ Calculated from osmotic pressure measurements in alcohol-water solutions, assuming the ideal solution law to apply.

|||| Cohn (6) gave this value as 48,000 on the basis of the writer's data.

¶¶ Test made with pepsin crystallized according to Northrop (28); the solution was saturated with $MgSO_4$.

*** This value is inferred from the note of Steinhardt (29), who stated that urea caused no dissociation of pepsin.

structure would not increase the number of molecules in a protein solution.

The gross structure of a protein containing 1 molecule of cystine in straight chain structure might be represented diagrammatically by the letter H, where the cross-bar corresponds to the disulfide bond, and the vertical bars to the peptide chains. In the conversion of S—S groups into —SH groups in a protein containing the straight chain type of structure of cystine, the molecular weight of the protein will be reduced. In the event that cystine occupies a terminal position in the molecule, then we shall represent diagrammatically its gross structure by the letter L.

3. That the straight chain and cyclic forms of cystine in proteins differ in stability. The stability of the cyclic form is such that no rupture of the disulfide bond occurs in urea solution. The stability of the straight chain structure in proteins is such that conversion into the cysteine form occurs in urea solution.

The above hypothesis is now applied to the results.

Group I—Egg albumin conforms to the observations and data of Table V on the assumption of the existence of its cystine, in part or entirely, in straight chain type of structure in the terminal (or near terminal) position (protein Type L). Hence, in urea solution, splitting is assumed to take place, active —SH groups form, and the molecular weight, although it changes by the weight of one or several amino acids, appears the same in urea as in water when determined by the osmotic pressure method. It is noted (Column 8 of Table V) that the egg albumin molecule contains but 1 or 2 molecules of cystine. Hence, all of its cystine can occupy terminal positions in the protein molecule.

Group II—The cystine in the proteins in this group appears to be present in part in the straight chain type of structure (protein Type H). Hence, in urea solutions, these proteins are ruptured, and show formation of —SH groups and reduction of molecular weight. It is seen from Column 8 of Table V that the cystine content of the dissociated proteins is in no case less than 0.5 mole. The fact that each molecule of dissociated protein contains at least 1 molecule of cysteine, or other form of reducible sulfur, offers strong support for the view that the sulfur linkages are involved in the isoelectric disintegration of these proteins.

The fact that certain proteins in Group II contain more cystine than the theoretical minimal value of 0.5 mole would imply, in accordance with the hypothesis proposed, either that (1) a certain proportion of the cystine in the dissociated proteins is present in the cyclic structure, or that (2) the dissociated products are connected together in the intact protein by more than one disulfide link, in which case the diagrammatic representation of such a protein in the simplest case would then be by the letter H with more than one cross-bar.

Group III—The proteins in this group are in harmony with the data in Table V on the assumption that all their cystine is present in the stable cyclic form. Hence, in urea solution there is no rupture of their disulfide bonds, no formation of —SH groups, and no change in molecular weight.

In order to obtain further evidence on the question of the existence of cyclic cystine, osmotic pressure measurements were carried out upon urea solutions of a representative protein of Group III, in which —SH formation was induced by reduction of the protein S—S groups with Na_2SO_3.[3] It follows that, if cystine is present as part of a cycle, no reduction in molecular weight of the protein should occur when —SH groups form in such proteins.

The experiments were carried out upon crystalline serum albumin.[4] The reduction of its disulfide to —SH was qualitatively shown by a strongly positive test with phosphotungstic acid. The results are given in Table VI. For comparison, measurements of the osmotic pressure of solutions of serum albumin in urea in the absence of a reducing agent are also given in Table VI. It is seen from Table VI that the osmotic pressure is definitely no greater for urea solutions of serum albumin which show the presence of —SH groups than in those in which —SH groups are not present. The cystine in serum albumin is therefore probably all in cyclic form.

The effect of reduction upon urea solutions of serum albumin was to cause marked changes in its properties. The approximately 2 per cent solutions of reduced serum albumin in Experiments

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[3] Clarke (30) has shown that the reaction between sulfite and cystine produces rupture of the disulfide bond with formation of —SH:

$$R—S—S—R + NaHSO_3 = R—SH + R—S—SO_3Na$$

[4] These experiments were completed in connection with earlier work (2).

185 and 186, after standing for a while upon their removal from the osmometers, were soft gels. These gels possessed thixotropic properties: by mere shaking they were transformed to the fluid state and set again to a gel after a few minutes. Normal serum albumin in urea solution in the absence of reduction does not show these effects. The properties of such reduced serum albumin solutions very much resemble those of the muscle globulin described by von Muralt and Edsall (31). Stern and White (32) have also noted an exceptionally marked increase in viscosity when the protein, insulin, in aqueous solution, was reduced and denatured by means of thioglycolic acid.

TABLE VI

Osmotic Pressure of Reduced and Normal Serum Albumin in Urea Solution near the Isoelectric Point

Solution 1. Solvent, 6.66 M urea, 0.087 M Na_2SO_3, 0.082 M HCl, 0.087 M acetate buffer.

Solution 2. Solvent, 6.66 M urea, 0.05 M acetate buffer.

Solution 3. Solvent, 6.66 M urea, 0.1 M Na_2SO_3, 0.094 M HCl.

Solution 4. Solvent, 6.66 M urea, 0.1 M Na_2SO_4, acetate buffer 0.1 N in Na acetate.

Solution No.	Serum albumin solution	Experiment No.	Temperature	Test for —SH group	pH	C Concentration per 100 cc. solvent	P Osmotic pressure	$\frac{P}{C}$
			°C.			gm.	cm. H_2O	
1	Reduced	60	25	+	5.8*	1.93†	5.32	2.75
2	Normal		0	−	5.8	1.93‡	6.08‡	3.15
3	Reduced	185	25	+	5.3§	5.4‖	27.18	4.72
4	Normal	186	25	−	5.3	5.48	25.42	4.96

* This value refers to the pH of the urea-buffer solution in the absence of neutralized Na_2SO_3.

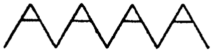
† In this experiment, before the colorimetric determination of protein concentration was performed, 20 cc. of 0.02 M acetic acid were added to the sample taken for analysis and the solution evaporated to a moist solid in an electric oven. This procedure destroyed the Na_2SO_3 present by oxidation. The same procedure was applied to the standard which was also prepared with Na_2SO_3 similar to the unknown.

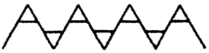
‡ These figures are taken from the corrected curve in Fig. 2 in (2).

§ Determined colorimetrically.

‖ Concentration of protein in solution as initially made up.

The high content of cystine in serum albumin (6 per cent) and in insulin (8 to 9 per cent) suggests that the reduction of this amino acid in certain proteins provides a mechanism for enhanced viscosity. Such enhanced viscosity after reduction of denatured serum albumin is consistent with the view that proteins in Group III have a structure containing peptide cycles of which cystine is a part. Since serum albumin is fairly rich in cystine it must contain a large number of such cycles. A diagrammatic representation of its structure in the native or denatured but unreduced state, in some simple fashion as by a series of connected A's, or alternately inverted A's,





is still consistent with the peptide hypothesis developed some 30 years ago by Hofmeister and by Fischer. After reduction of the S—S groups (cross-bars), the protein exhibits a much more extended form. This would explain the increase in viscosity without appreciable change in molecular weight. For according to the recent conclusions of Mirsky and Pauling (33), "The increase in viscosity of protein solutions . . . we attribute to the change from the compact configuration . . . to more extended configurations."

Concluding Remarks—The results of the experiments reported in this paper indicate that protein instability at the isoelectric point is not primarily determined by the formation of active —SH groups in the protein. Empirically it has been found that typically denaturable proteins, whose molecular weights are greater than 1 × 34,500, will show instability in urea solution, if their dissolution in this solvent causes the spontaneous or direct formation of —SH groups. And conversely, proteins in isoelectric urea solution, which do not directly form —SH groups, will show stability toward changes in molecular weight.

SUMMARY

1. From measurements of the osmotic pressure upon amandin and excelsin in aqueous buffer and glycerol solutions near the isoelectric point, the mean molecular weights were found to be 206,000 and 214,000 respectively. These values agree fairly well

with those obtained by Svedberg and Sjögren by the ultracentrifugal method.

2. From osmotic pressure measurements in urea solutions near the isoelectric point, the mean molecular weights of amandin and excelsin were found to be 30,300 (provisional value) and 35,700, respectively.

3. In urea solution, amandin and excelsin show directly the presence of —SH groups. Such groups are not apparent in the proteins when dissolved in aqueous buffer or glycerol solutions.

4. Comparison of the molecular weights of nine other proteins in aqueous and urea solutions with qualitative tests for the presence of —SH groups indicates a certain degree of correlation to exist between the instability (or stability) of a protein at the isoelectric point and the activity of its sulfur linkages.

5. The results have been explained by assuming cystine to exist in proteins in two types of structure, a straight chain form and a cyclic form.

BIBLIOGRAPHY

1. Burk, N. F., and Greenberg, D. M., *J. Biol. Chem.*, **87**, 197 (1930).
2. Burk, N. F., *J. Biol. Chem.*, **98**, 353 (1932).
3. Hopkins, F. G., *Nature*, **126**, 328, 383 (1930).
4. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **18**, 307 (1934–35); **19**, 427 (1935–36).
5. Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, **106**, 605 (1934); **112**, 361 (1935–36).
6. Cohn, E. J., *Ergebn. Physiol.*, **33**, 781 (1931).
7. Calvery, H. O., and Freyberg, R. H., *J. Biol. Chem.*, **109**, 739 (1935).
8. Svedberg, T., and Sjögren, B., *J. Am. Chem. Soc.*, **52**, 279 (1930).
9. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
10. Greenberg, D. M., *J. Biol. Chem.*, **82**, 545 (1929).
11. Cohn, E. J., *J. Am. Chem. Soc.*, **49**, 173 (1927). Cohn, E. J., Heyroth, F. F., and Menkin, M. F., *J. Am. Chem. Soc.*, **50**, 696 (1928).
12. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin (1914). Walpole, G. S., *J. Chem. Soc.*, **105**, 2501 (1914).
13. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition (1928).
14. Csonka, F. A., Murphy, J. C., and Jones, D. B., *J. Am. Chem. Soc.*, **48**, 763 (1926).
15. Adair, G. S., and Callow, E. H., *J. Gen. Physiol.*, **13**, 819 (1929–30).
16. Adair, G. S., and Robinson, M. E., *Biochem. J.*, **24**, 1864 (1930).
17. Sörensen, S. P. L., *Z. physiol. Chem.*, **106**, 1 (1919); *Compt.-rend. trav. Lab. Carlsberg*, **12**, 262 (1917).

18. Heffter, A., *Med. naturwissensch. Arch.*, **1**, 81 (1907); *Chem. Zentr.*, **11**, 882 (1907). Arnold, V., *Z. physiol. Chem.*, **70**, 300 (1911). Harris, L. J., *Proc. Roy. Soc. London, Series B*, **94**, 426 (1923). Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).
19. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 109 (1929).
20. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
21. Svedberg, T., and Nichols, J. B., *J. Am. Chem. Soc.*, **49**, 2920 (1927).
22 Vickery, H. B., and White, A., *J. Biol. Chem.*, **99**, 701 (1932–33).
23. Weber, H. H., and Stöver, R., *Biochem. Z.*, **259**, 269 (1933). Weber, H. H., *Ergebn. Physiol.*, **36**, 109 (1934).
24. Mirsky, A. E., *J. Gen. Physiol.*, **19**, 559 (1935–36).
25. Svedberg, T., and Stamm, A., *J. Am. Chèm. Soc.*, **51**, 2170 (1929).
26. Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, **62**, 183 (1924–25).
27. Burk, N. F., unpublished measurements.
28. Northrop, J. H., *J. Gen. Physiol.*, **13**, 767 (1929–30).
29. Steinhardt, J., *Nature*, **138**, 800 (1936).
30. Clarke, H. T., *J. Biol. Chem.*, **97**, 235 (1932).
31. von Muralt, A. L., and Edsall, J. T., *J. Biol. Chem.*, **89**, 315 (1930).
32. Stern, K. G., and White, A., *J. Biol. Chem.*, **117**, 95 (1937).
33. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, **22**, 439 (1936).

A COMPARISON OF THE HYPERVITAMINOSES INDUCED BY IRRADIATED ERGOSTEROL AND FISH LIVER OIL CONCENTRATES*

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It is now generally recognized that the D provitamins of ergosterol and fish liver oils are not identical. The work of Waddell (11) and of numerous other investigators on the comparative antirachitic effectiveness of irradiated ergosterol, irradiated cholesterol, and fish liver oils for rats and chicks has disclosed markedly varying minimum curative levels for these substances.

Few studies have been reported, however, covering the effects of excessive dosage of these vitamin D sources. Dalmer, von Werder, and Moll (4), prepared the non-saponifiable portions of cod, tuna, and halibut liver oils, removed cholesterol, and treated the residue with maleic anhydride to remove the vitamin A. They stated that the vitamin D remaining was unimpaired and, when compared with irradiated ergosterol, was found to have the same antirachitic and also toxic properties. The toxic quality of the preparations was judged by determining the minimum lethal dose for mice. The ratio of toxic to antirachitic doses was said to be of the same order in the treated liver oil preparations as in irradiated ergosterol. Evidently these investigators reasoned that the large vitamin A content of liver oils might add to the toxicity of excessive vitamin D values.

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* Part of this study was reported at The Fifteenth International Physiological Congress at Leningrad, August 14, 1935.

Assistance in the latter part of this investigation was rendered by Works Progress Administration Project No. 2445, assigned to the University of California.

The work of Brockmann (3) on the isolation of a substance from tuna liver oil which corresponds in chemical as well as biological properties with the irradiated 7-dehydrocholesterol of Windaus, Schenck, and von Werder (12), appears to confirm the impression that this liver oil at least contains an antirachitic vitamin which is different from calciferol. There is a good deal of evidence, first pointed out by Bills, Massengale, and Imboden (2), that tuna and other fish liver oils may contain one or more antirachitic vitamins different from that of cod liver oil as well as different from calciferol.

The present inquiry deals with the differences which may exist in the response of young rats to the administration of carefully measured excessive doses of irradiated ergosterol and fish liver oil concentrates. The doses were measured so as to be equivalent in international units of vitamin D. The further question arose as to the effect on the hypervitaminoses of varying amounts of vitamin A administered along with the vitamin D-rich supplements. Gross-Selbeck (5) reported that alleviation of hypervitaminosis D resulted from the giving of large but subtoxic amounts of vitamin A, and Thoenes (10) in a review of this field came to the same conclusion.

Another question concerned the possible effect of sex upon severity of hypervitaminosis D. Kern, Montgomery, and Still (6) reported the kidney ash of male rats fed irradiated ergosterol at the levels of 50,000 to 100,000 D much more increased than was that of females treated similarly. Agduhr (1) likewise reported male mice as more susceptible to poisoning by irradiated ergosterol than the females when the animals were kept separately. When caged in pairs, a curious fall in the mortality of the males occurred and an unusual number of pregnancies with no mortality was seen in the females.

The procedures used in the study here reported were designed to obtain new evidence on all three of these questions. Rats were taken at 28 days of age, caged separately on screens, and given an adequate basal diet of normal calcium and phosphorus content and ratio. This diet which has been used in previously reported comparable studies (8, 9) on the effects of vitamin D and parathyroid extract has the following composition.

	per cent
Wheat gluten	10
Dried egg albumin	10
Agar	2
Crisco	15
Corn-starch	59
Salt mixture (8)	4

Its calcium content was 0.45 to 0.65 per cent and phosphorus, 0.35 to 0.50 per cent. In some of the experiments the B vitamins were given in 100 mg. daily supplements of yeast extract (Harris) and in some as 0.5 gm. of dry brewers' yeast (Northwestern). The vitamin A was supplied in a coconut or corn oil solution of pure carotene or in the fish liver oils and concentrates. All vitamin A- and vitamin D-bearing supplements were given orally by pipette.

In all experiments complete control series were included with litter mates, distributed so as to provide comparable records on each preparation fed. The response of each group of animals was judged by the performance of the normally fed controls included within the same experiment. The rate of growth, ash content of the femurs, serum calcium and inorganic phosphorus, and ash, calcium, and phosphorus content of the soft tissues, particularly of the kidneys, were determined in all cases at the end of the feeding periods, which varied from 21 to 57 days. In three of the experiments representative animals were sacrificed after 14, 28, 43, and 57 days on the diet in order to follow the progress of the changes induced. Representatives of each litter were sacrificed at the beginning of the experiments in most cases to establish the blood, bone, and organ composition of the animals at the start.

The femurs were dried, extracted with alcohol and ether in the usual fashion, and ashed at 450°. Femurs from two to four rats of the same group were ashed together usually. The blood samples of the same rats were pooled for calcium and inorganic phosphorus determinations of serum. The lungs, hearts, kidneys, and livers were dissected at once from the carcasses, pooled in the same groups used for blood and bones, weighed fresh, dried, weighed, and ashed at 450°. The later calcium determinations[1]

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[1] We acknowledge with thanks the assistance in these analyses of Carroll A. Handley, assigned to Works Progress Administration Project No. 2445 at the University of California.

Effect of Hypervitaminosis D from 10,000 Units Daily of Irradiated Ergosterol upon Male and Female Rats

Experiment No.	Group No.	Vitamin source	Units daily		No. of rats	Sex	Weight		Length of test	Serum		Femur ash	Per cent of dry weight							
													Liver		Lung		Heart		Kidney	
			Vitamin D	Vitamin A			At beginning (29 days)	Gain		Ca	P		Ca	P	Ca	P	Ca	P	Ca	P
							gm.	*gm.*	*days*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*								
IV	1	Irradiated ergosterol	8		6	M.	55	120	35	11.9	10.7	56.7	0.030	0.68	0.08	0.51	0.09	0.54	0.04	0.70
		Carotene		10	6	F.	51	80	35	11.6	11.0	58.4	0.018	0.65	0.09	0.69	0.10	0.66	0.05	0.71
	2	Irradiated ergosterol	10,000		6	M.	67	56	35	15.2	8.4	45.5	0.021	0.60	0.07	0.63	0.14	0.60	0.15	0.78
		Carotene		200	6	F.	67	36	35	15.0	8.1	48.9	0.018	0.60	0.16	0.65	0.22	0.63	0.25	0.93
	3	Irradiated ergosterol	10,000		4	M.	53	107	37	14.0	11.2	52.3	0.052	0.84	0.08	0.70	0.08	0.69	0.19	0.93
		Halibut liver oil	24	2500	4	F.	52	55	37	16.2	9.5	49.0	0.042	0.79	0.21	0.77	0.14	0.69	0.23	0.89
	4	Irradiated ergosterol	10,000		4	M.	57	31	28	14.0	8.9	43.3	0.056	0.84					1.66	1.45
		Carotene		200	2	F.	50	26	28	15.4	8.3	45.9	0.052	0.88					1.64	2.05
	5*	Irradiated ergosterol	10,000		4	M.	55	−3	9			45.2	0.059	0.87					5.40	2.76
		Carotene		200	3	F.	50	−5	10			44.4	0.018	0.99	0.57	1.00	0.88	1.28	1.57	1.98
	6*	Irradiated ergosterol	10,000		4	M.	53	14	17			41.4	0.026	1.08	1.01	1.14	1.27	1.56	3.13	3.27
		Carotene		10	3	F.	50	−3	10			45.3	0.013	0.87	0.63	1.07	0.99	1.47	2.23	2.30
V	1	Cod liver oil	8	250	4	M.	46	114	37	14.3	8.4	52.5			0.06		0.12		0.04	
					2	F.	46	104	37	15.0	8.1	53.5			0.13		0.10		0.05	
	2	Irradiated ergosterol	10,000		8	M.	52	11	37	19.9	9.2	40.3			0.33		0.68		0.50	
		Carotene		200	7	F.	49	17	37	16.6	9.2	43.2			0.90		0.65		0.35	

* These rats died of overdosage.

on ash of serums and viscera were made by a microfiltration modification of the permanganate titration method (7), but those recorded in Experiments I to IV as well as the phosphorus analyses were done by the methods previously described (8, 9).

All the oils and concentrates for vitamin D and vitamin A were assayed repeatedly, by the official United States Pharmacopœia (1935) method. Vitamin A potency as judged by the antimony trichloride blue values of the oils was determined also by the technique prescribed in the British Pharmacopœia (1932).

Eight experiments were carried out in all. In Experiments I, II, and III groups of four to twelve animals were sacrificed after 14, 28, 43, and 57 days on each of the concentrates. Experiments IV and V (Table I) were conducted with irradiated ergosterol only as the source of excessive vitamin D; the amounts of vitamin A were varied and separate examinations made of male and female animals. Experiments VI, VII, and VIII (Table II) were designed to bring out the varying effects of two or three different sources of both vitamins D and A.

In Experiment I four groups were used, Group 1 being a control lot of rats given 8 units of vitamin D and 10 units of vitamin A daily in the form of a tuna liver oil[2] assayed as having 50,000 units of vitamin D and 61,000 of vitamin A per gm.[3] Group 2 was given 4000 units of vitamin D and 990 units of vitamin A daily from the same tuna liver oil. Group 3 was given daily 4000 units of vitamin D and 9200 units of vitamin A as a highly concentrated extract of cod liver oil, 103,000 units of vitamin D per gm., prepared and assayed by the laboratory of the National Oil Products Company.[4] Group 4 received 4000 units of vitamin D daily as an irradiated ergosterol preparation assayed to contain 1,000,000 units per gm. and carotene at the level of 0.02 mg. daily as source of vitamin A.

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[2] The tuna liver oil, one of the samples of halibut liver oil, the irradiated ergosterol, and part of the calciferol used in this study were all supplied through the courtesy of Dr. C. E. Bills of Mead Johnson and Company, Evansville, Indiana. The vitamin values of these preparations were given us by Dr. Bills, to whom we wish to express our gratitude.

[3] All units of vitamins A and D mentioned in this paper are the international (or U.S.P.) units.

[4] We desire to express our gratitude to Mr. A. V. Swarthout and to the National Oil Products Company of Harrison, New Jersey, for this and for other generous cooperation without which this study could not have been completed.

TABLE II

Biochemical Data Obtained on Rats Given Excess Amounts of Vitamins D and A As Irradiated Ergosterol, Fish Liver Oils, and Carotene

Experiment No.	Group No.	Vitamin source	Units daily		No. of rats	Weight		Length of test	Serum		Femur ash	Ca, per cent of dry weight		
			Vitamin D	Vitamin A		At beginning	Gain		Ca	P		Lung	Heart	Kidney
						gm.	*gm.*	*days*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*			
VI	1	Cod liver oil	8	250	12	50	69	27	12.4	9.1	52.2		0 136	0.170
	2	Carotene	0	10	8	45	68	27	10.7	9.6	52.9	0.206	0.114	0.165
	3	Tuna liver oil concentrate	1,200	300	6	52	59	27	10.7	9.4	51.9	0.207	0.251	0.157
		Halibut liver oil	47	10,000										
	4	Irradiated ergosterol	10,000		12	50	28	27	12.9	8.9	45.7	0.678	0.357	0.471
		Halibut liver oil	47	10,000										
	5	Irradiated ergosterol	10,000		10	50	27	27	13.7	8.4	44.0	1.284	0.681	1.280
		Halibut liver oil		280										
	6	Irradiated ergosterol	10,000		10	50	2	27	15.5	7.1	45.5	2.700	0.802	1.302
		Carotene		100										
VII	1	Weanling controls			7	46	0	0	11.2	11.6	41.1	0 125	0.065	0.093
	2	Cod liver oil	8	250	7	46	80	21	11.5	4.9	58 5	0.143	0.135	0.059
	3	Striped tuna liver oil concentrate	10,000	3,300	7	48	44	21	12.8	5.8	52 8	0.143	0.136	0.095
		Halibut liver oil	47	10,000										
	4	Striped tuna liver oil concentrate	10,000	3,300	8	45	48	21	13.3	6.4	53.2	0.162	0.074	0.161
		Carotene		100										
	5	Irradiated ergosterol	10,000		7	43	40	21	13.1	5.3	52.1	0.166	0.457	0.247
		Halibut liver oil	47	10,000										
	6	Irradiated ergosterol	10,000		7	45	21	21	14.1	5.0	52.6	2.258	0.270	1.040
		Carotene		100										
	7	Calciferol	10,000		4	48	24	21	12.0	4.6	46.7	0.682	0.260	0.584
		Carotene		100										

VIII	1	Weanling controls			8	50	0	0	16.1	8.0	44.2	0.300	0.321	0.103
	2	Cod liver oil	8	250	7	50	64	31	11.2	10.2	53.1	0.110	0.117	0.076
	3	Tuna liver oil concentrate	1,400	1,200	19	50	57	31	15.3	9.6	53.0	0.152	0.103	0.086
		Halibut liver oil	18	3,800										
	4	Cod liver oil concentrate	10,300	23,000	5	52	58	31	17.2	9.2	52.2	0.421	0.316	0.173
	5	Irradiated ergosterol	10,000		12	49	52	31	15.2	9.5	49.2	0.218	0.191	0.264
		Halibut liver oil	18	3,800										
	6	Irradiated ergosterol	10,000		19	49	43	31	14.9	9.0	44.0	0.617	0.221	0.606
		Carotene		1,100										

In Experiment II four groups also were used, the controls (Group 1) being given daily 8 units of vitamin D and 250 units of vitamin A in the form of the U.S.P. reference cod liver oil. Groups 2 and 3 were given respectively 100 and 250 units of vitamin D daily as a concentrate of white sea-bass liver oil, prepared and assayed in this laboratory,[5] which carried 2000 and 5000 units of vitamin A. Group 4 received 10,000 units of vitamin D daily as irradiated ergosterol and 0.02 mg. of carotene or 20 units of vitamin A. Some of each of these groups were killed at the end of 14, 28, and 43 days on the diet. Four of the ergosterol group died of overdosage after 17 to 35 days on the diet.

In Experiment III the control (Group 1) and sea-bass liver oil (Group 2; 250 units of vitamin D daily) were also included and two groups receiving 10,000 units of vitamin D daily from irradiated ergosterol, Group 3 being given in addition 24 units of vitamin D and 2500 units of vitamin A in the form of halibut liver oil, and Group 4, only 40 units of vitamin A as halibut liver oil. Again some animals were killed after 14, 28, and 43 days on the diet.

The growth in all three of these experiments is shown in Fig. 1. Obviously the irradiated ergosterol impeded growth the most in all three cases, the control animals on low vitamins D and A grew best, and the moderate vitamin D content, 100 and 250 units daily, of the sea-bass liver oil interfered but little with growth.

The serum calcium of the rats in all three experiments is shown in Fig. 2. Here again a progressive rise is noted as the medication continued, with the largest values in all cases shown by groups receiving irradiated ergosterol. The fish liver oil concentrates produced less striking but consistent hypercalcemia.

The per cent of ash in the dry extracted femurs is shown in Fig. 3. In all cases the lowest ash was found in the irradiated ergosterol groups, but the discrepancy among the groups in Experiment I (4000 units daily) was much less striking than in Experiments II and III (10,000 units daily).

Kidney calcium expressed as per cent of dry tissue for the rats of Experiments I and II is shown in Fig. 4. A continuous rise occurred in all hypervitaminotic rats, with the most striking

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[5] Prepared from livers of white sea-bass (*Cynoscion macdonaldi*) freshly caught in southern Californian waters and received in the laboratory from the National Oil Products Company in the early spring of 1935.

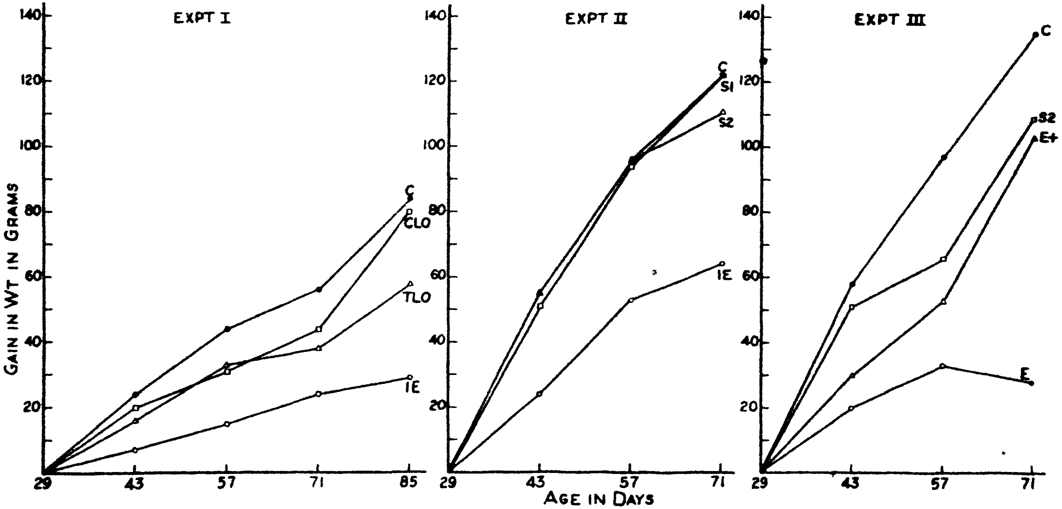

FIG. 1. Growth of young rats fed excessive amounts of vitamin D and vitamin A from different sources. Experiment I, *C*, normal controls; *CLO*, 4000 units of vitamin D daily from cod liver oil concentrate; *TLO*, same from tuna liver oil; *IE*, same from irradiated ergosterol. Experiment II, *C*, normal controls; *S1*, 100 units of vitamin D daily from sea-bass liver oil; *S2*, 250 units of vitamin D daily from sea-bass liver oil; *IE*, 10,000 units of vitamin D daily from irradiated ergosterol. Experiment III, *C*, normal controls; *S2*, 250 units of vitamin D daily from sea-bass liver oil; *E* +, 10,000 units of vitamin D daily from irradiated ergosterol, and 2500 units of vitamin A from halibut liver oil; *E*, 10,000 units of vitamin D daily from irradiated ergosterol and 40 units of vitamin A from halibut liver oil.

increase in the irradiated ergosterol groups. Definite increase is seen also in kidney calcium of the tuna liver oil and sea-bass liver oil groups. Lung and heart, as well as kidney calcium for the animals of Experiment III, are shown in Fig. 5. Large increases

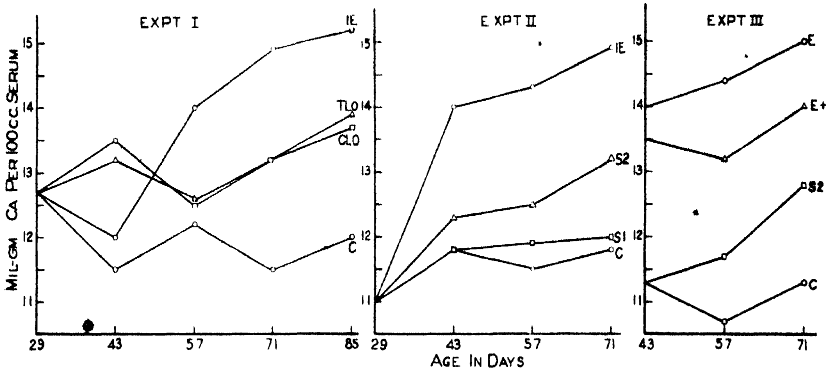

FIG. 2. Serum calcium as affected by various amounts of different sources of vitamin D. Groups designated as in Fig. 1.

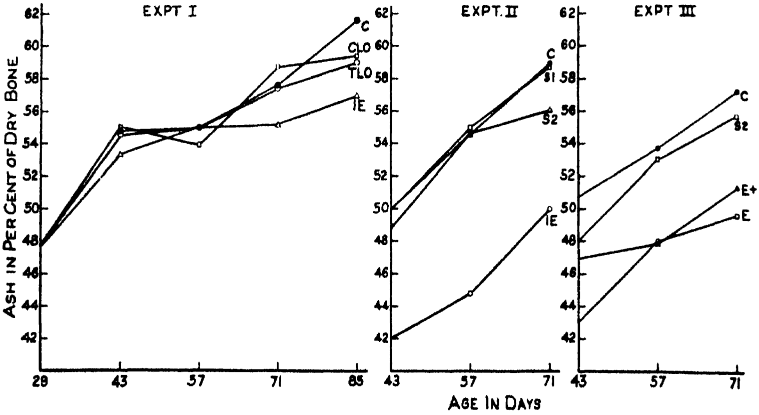

FIG. 3. Ash content of femurs as affected by various doses of different sources of vitamin D. Groups designated as in Fig. 1.

occurred only in the groups fed irradiated ergosterol, with maxima at 28 days on the diet. Possibly the greater size attained by the rats following this period decreased the severity of the calcification produced by the constant vitamin D dosage.

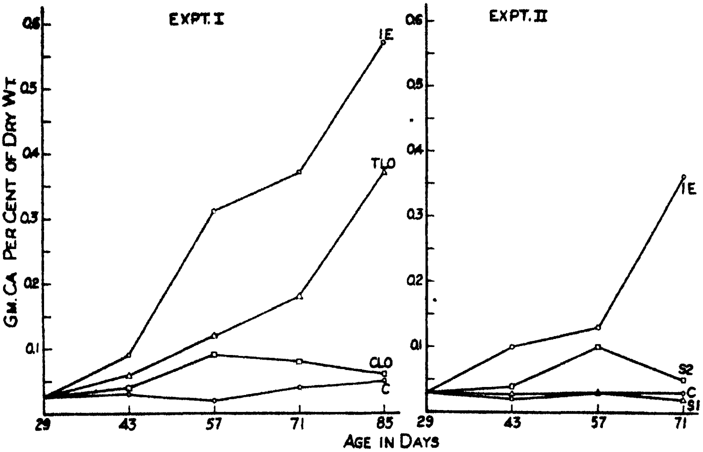

FIG. 4. Calcium content of kidneys of rats as affected by various doses of different sources of vitamin D. Groups designated as in Fig. 1.

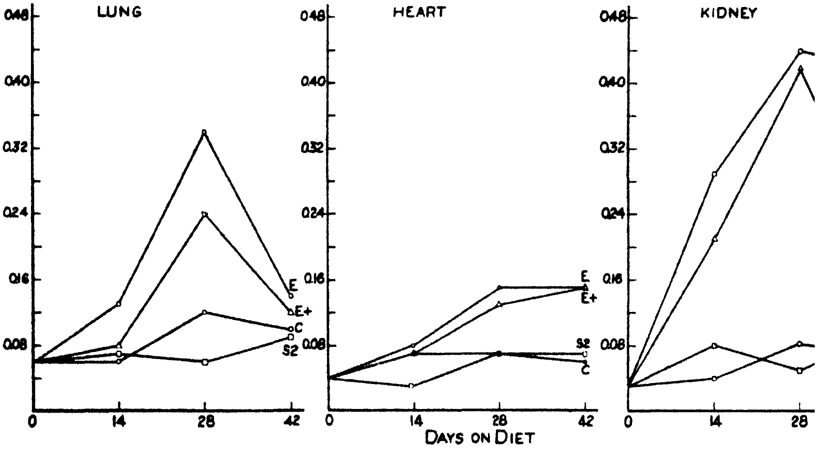

FIG. 5. Lung, heart, and kidney calcium content of rats as affected by 10,000 units of vitamin D daily from irradiated ergosterol with varying vitamin A intake. Experiment III, groups designated as in Fig. 1.

Toxicity of Irradiated Ergosterol As Compared with That of Fish Liver Oil

Groups 2, 3, and 4 of Experiment I, Groups 3 to 7 of Experiment VII, and Groups 4, 5, and 6 of Experiment VIII may fairly be compared. In every respect, as shown in Figs. 1 to 4, the rats given irradiated ergosterol in Experiment I were more severely affected than were those given the liver oils. This is particularly striking in the rise of the calcium content of the kidneys of the former groups. It is plain also that the group given the excess dosage of tuna liver oil deviated somewhat more from the normal as to growth and kidney calcium than did those receiving the cod liver oil concentrate.

In Experiment VII (Table II), the groups which received the tuna liver oil concentrate were better off than those which received either irradiated ergosterol or crystalline calciferol.[6] Groups 4 and 5, which are fairly comparable as to both vitamin D and vitamin A intake, indicate somewhat better growth, femur ash, and organ calcium content in the former (tuna liver oil concentrate group), even though the vitamin A intake was the smaller. Groups 6 and 7, with relatively low carotene and calciferol as source of vitamin D, show decidedly more toxic conditions than do any of the other animals. The results for these two groups, incidentally, appear to indicate that the calciferol of the irradiated ergosterol is solely responsible for the toxic effects observed.

In Experiment VIII, Group 4, which received the cod liver oil concentrate (Vitex concentrate), showed superior growth, femur ash, and kidney calcium, but heart and lung calcium were decidedly increased. There is little doubt, however, that the comparable rats which received irradiated ergosterol were more unfavorably affected by their regimen than were any others in this series.

Effect of Vitamin A upon Toxicity of Excess Vitamin D

In the first experiments it was assumed that vitamin A administration equivalent to that found adequate in the control diets without excess vitamin D would exert the necessary protection in the groups fed excess vitamin D. The advantage enjoyed by the cod liver oil-fed group in Experiment I over that given tuna liver oil led to the suspicion that the large vitamin A content of the cod liver oil concentrate might be exerting some protective effect. In Experiment III, therefore, halibut liver oil was given at two levels to Groups 3 and 4 along with 10,000 units of vitamin

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[6] Part of the crystalline calciferol was supplied by the Winthrop Chemical Company, through the courtesy of Dr. O. W. Barlow.

D in irradiated ergosterol, Group 3 receiving 2500 units of vitamin A daily and Group 4, 40 units. The amount of vitamin D added in the halibut liver oil to the two latter groups was considered negligible. Group 3 grew better, had more femur ash, and lower organ calcium at each stage at which animals were sacrificed.

In Experiment VI Group 4, receiving 10,000 units of vitamin A daily, may be compared with Groups 5 and 6 receiving only 280 or 100 units, all receiving an equal excess of vitamin D. While the relatively large vitamin A intake of Group 4 did not protect entirely against symptoms of hypervitaminosis, it apparently prevented the extreme calcification of lungs, heart, and kidneys seen in Groups 5 and 6. One of Group 4, two of Group 5, and four of Group 6 died before the end of the test period.

In Experiment VII, Groups 6 and 7 with low vitamin A may be compared with Group 5 which had 10,000 units of vitamin A daily, to the considerable advantage of the latter. Again Group 3 may be compared with Group 4, both having 10,000 units of vitamin D and 3300 units of vitamin A daily from striped tuna liver oil concentrate, and the former an additional 10,000 units of vitamin A from halibut liver oil. Again the advantage, although this time not a notable one, rests with the large vitamin A intake. Certainly no evidence of unfavorable effect from this relatively huge excess of vitamin A could be seen during the short period of the observation. Only one animal, one of Group 7, died before the end of the period in this experiment.

In Experiment VIII Group 6, which had the lower intake of vitamin A along with excess irradiated ergosterol, developed greater calcification of the soft tissues, decalcification of the bones, and less growth than did Group 5, which received the larger amount of vitamin A.

The better growth achieved by young rats fed large amounts of vitamin A may be thought to account for the lessening of the unfavorable symptoms of hypervitaminosis D. The last two groups compared, however, had relatively little difference in final average weights but marked differences in the other criteria examined. Group 4 in Experiment VII grew less than Group 3, but the former with high vitamin A intake approximated the condition of the controls more nearly than did the latter. Groups 4 and 5 in Experiment VI offer another example of groups gaining nearly the same amount in weight but definitely better conditions are seen in the animals on large vitamin A intake.

The slackening of growth with decreased food intake is itself, of course, part of the syndrome of hypervitaminosis D, an effect which cannot fairly be dissociated from the other unfavorable symptoms. There is sufficient evidence, however, in the experiments described, whether weight gains be equalized or not, to justify the conclusion that increased intake of vitamin A has a definitely protective action against the toxic effect of excessive intake of vitamin D. So far as these experiments went, no limitation to this effect was found, although the vitamin A doses used varied from 10 to 23,000 units daily.

Effect of Sex on Hypervitaminosis D

As shown in Table I, all groups in Experiments IV and V were separated as to sex. In nearly all cases the average weight of the males was greater both at the beginning and at the end of the observation period. The serum calcium was sometimes less and sometimes greater in the males, but the femur ash was usually greater in the females. More calcification of the soft tissues occurred in the females of Groups 2 and 3, Experiment IV, but the opposite was the case in Group 2 of Experiment V. The rats which died of the overdosage (Groups 5 and 6, Experiment IV) had equal and very extensive calcification of all the organs examined.

It seems fair to conclude that the case is not proved for any specific sex sensitivity to hypervitaminosis D. A summary of the data bearing on this question is given in Table III.

The increase in phosphorus content of the organs found throughout the whole study was somewhat inconsistent and apparently not significant, owing perhaps to the relatively large normal phosphorus content of these tissues, except when the most advanced metastic calcification had occurred. Such was the case in Groups 4, 5, and 6 of Experiment IV. The calcium and phosphorus of the liver were not greatly changed in any of the groups studied, even in those which showed extensive alteration of the normal composition of the other organs.

It has sometimes been suggested that the rise in calcium content of the kidney tissue of hypervitaminotic rats may be due to the large calcium content of the urine secreted. In Table IV the calcium content of the undiluted urine of certain animals used in Experiment VIII is compared with the calcium content of fresh kidney tissues of the same groups. The largest urinary calcium excretion was that of the group fed cod liver concentrate, whereas the kidney calcium in this group was only approximately double that of the normal animals. The groups receiving irradiated ergosterol, with greater kidney calcium, excreted urines lower in calcium. This may be interpreted to mean either that the excretory power of the damaged kidneys in the latter case was decreased or that the calcium deposition in the kidney tissue was independent of urine concentration.

TABLE III

Summary of Sex Effect upon Hypervitaminosis D

No. of rats	Sex	Vitamin D per day	Femur ash	Ca, per cent of dry weight			Gain in weight	Length of test period
				Kidney	Heart	Lung		
		units	*per cent*				*gm.*	*days*
10	M.	8	54.6	0.045	0.154	0.072	117	36
8	F.	8	56.8	0.055	0.100	0.101	88	36
30	M.	10,000	44.1	1.547	0.498	0.338	35	29
25	F.	10,000	46.1	0.782	0.488	0.509	23	29

TABLE IV

Relation of Urinary Calcium to Kidney Calcium Content

Experiment VIII Group No.	Vitamin source	Vitamin D per day	Urinary Ca, mg. per cent urine		Kidney Ca, mg. per cent fresh tissue	
			Range	Mean	Range	Mean
		units				
2	Cod liver oil	8	2.3– 3.6	3.0	10– 37	20
3	Tuna liver oil concentrate (and halibut liver oil)	1,400	2.6– 8.3	5.1	10– 43	22
4	Cod liver oil concentrate	10,300	51.4–79.1	65.2	40– 46	43
5	Irradiated ergosterol (and halibut liver oil)	10,000	22.1–47.6	29.6	44– 86	64
6	Irradiated ergosterol (and carotene)	10,000	22.1–80.9	45.4	67–250	169

A summary of some of the comparable data along with the

TABLE V

Comparison of Irradiated Ergosterol and Fish Liver Oils As Productive of Hypervitaminosis D[*]

Group No.	No. of rats	Vitamin D		Vitamin A	Femur ash	Ca, per cent of dry weight			Gain in weight	Test period
		Source				Kidney	Heart	Lung		
			units per day	*units per day*	*per cent*				*gm.*	*days*
1	115	Cod liver oil or other	8	10–250	54.9±0.31	0.065±0.009	0.069±0.018	0.116±0.013	76	29
2	86	Fish liver oil concentrates	100–1400	2000–10,300	53.8±0.68	0.067±0.015	0.103±0.042	0.128±0.027	74	27
3	20	" "	10,000	3300–23,000	52.8±0.20	0.141±0.016	0.156±0.049	0.220±0.080	49	24
4	55	Irradiated ergosterol	10,000	2500–10,000	48.7±0.23	0.308±0.030	0.226±0.069	0.281±0.045	49	28
5	135	" "	10,000	20–1100	45.2±0 56	0 835±0.159	0.472±0.075	0.876±0.163	27	28

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[*] *Cf.* "Results."

probable errors of the means from the eight experiments is given in Table V. There are clearly significant differences in femur ash and organ calcium content between the normal animals on low vitamin D and A intake and all the rats on the high vitamin D intake, with the exception of the group given relatively small excesses of fish liver oil concentrate. The latter were normal by all criteria used. Significant and relatively large differences exist between the group given 10,000 units of vitamin D daily in fish liver oil concentrates and in those given the same in irradiated ergosterol with moderate vitamin A, but less striking although still statistically significant differences as to femur ash and kidney calcium occurred between the former and the group given the irradiated ergosterol and excess vitamin A.

The excess fish liver oil produced animals with femur ash lower than that of the controls and kidney calcium probably significantly higher, but with no clear effect upon heart and lung calcium.

If decrease in growth rate, lowering of femur ash, and increase in calcium content of the viscera be accepted as indications of undesirable effects of vitamin D administration, the data here presented demonstrate that in like amounts calciferol, the vitamin D of irradiated ergosterol, is more toxic to rats than the vitamin or vitamins of fish liver oils. Since, in addition, it appears that a large excess of vitamin A further depresses the toxic effect of excess vitamin D, additional protection is afforded by the natural vitamin A content of the liver oils. The clinical choice of vitamin D carrier, particularly whenever large amounts need to be given, might reasonably be governed by these considerations.

SUMMARY

Eight series of feeding tests, 21 to 57 days in length, were made on a total of more than 500 rats placed at weaning on a synthetic diet of normal calcium and phosphorus content. Comparable animals were given: (1) normal amounts of vitamins A and D (10 to 250 international units) daily, (2) 100 to 10,000 units of vitamin D as tuna, sea-bass, and cod liver oil concentrates, (3) 4000 or 10,000 units of vitamin D as irradiated ergosterol or crystalline calciferol, (4) in some of each of Groups 2 and 3 varying amounts of vitamin A (10 to 23,000 units daily).

Depression of growth, mortality, hypercalcemia, decrease in femur ash, and increase in calcium content of the viscera were all most marked in the animals given the irradiated ergosterol, 4000 or 10,000 units of vitamin D daily, and small additions of vitamin A-containing supplements. Those which received 10,000 units of vitamin D as irradiated ergosterol along with much increased vitamin A, 2500 to 10,000 units daily, had decreased but still obvious signs of hypervitaminosis D. Those which received the fish liver oil concentrates in all cases were nearly normal and in only one group showed evidence of hypervitaminosis. Male and female animals were apparently equally susceptible to the toxic action of irradiated ergosterol, the females usually maintaining a higher femur ash value but having more advanced calcification of the viscera.

CONCLUSIONS

1. In rats calciferol, the vitamin D of irradiated ergosterol, exerts greater toxic effects at lower levels than do the vitamins D of fish liver oil.
2. Male and female animals are nearly equally susceptible to hypervitaminosis D.
3. A large excess of vitamin A, 250 to 1000 times that needed under normal conditions, may decrease but does not eliminate the harmful results of excess irradiated ergosterol. Some advantage may accrue from the use of excess vitamin A also in animals given large amounts of vitamin D in fish liver oil concentrates.

BIBLIOGRAPHY

1. Agduhr, E., *Z. Vitaminforsch.*, **4**, 54 (1935).
2. Bills, C. E., Massengale, O. N., and Imboden, M., *Science*, **80**, 596 (1934).
3. Brockmann, H., *Z. physiol. Chem.*, **241**, 104 (1936).
4. Dalmer, O., von Werder, F., and Moll, T., *Z. physiol. Chem.*, **224**, 86 (1934).
5. Gross-Selbeck, C., *Klin. Woch.*, **14**, 61 (1935).
6. Kern, R., Montgomery, M. F., and Still, E. U., *J. Biol. Chem.*, **93**, 365 (1931).
7. Kirk, P. L., and Schmidt, C. L. A., *J. Biol. Chem.*, **83**, 311 (1929).
8. Morgan, A. F., Kimmel, L., Thomas, R., and Samisch, Z., *J. Biol. Chem.*, **106**, 531 (1934).
9. Morgan, A. F., and Samisch, Z., *J. Biol. Chem.*, **108**, 741 (1935).
10. Thoenes, F., *Deutsch. med. Woch.*, **61**, 2079 (1935).
11. Waddell, J., *J. Biol. Chem.*, **105**, 711 (1934).
12. Windaus, A., Schenck, F., and von Werder, F., *Z. physiol. Chem.*, **241**, 100 (1936).

STUDIES OF UREA, CREATININE, AND AMMONIA EXCRETION IN DOGS IN ACIDOSIS*

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Van Slyke, Page, Hiller, and Kirk (1) have reported, in recent studies on man, that when the proportion of urea in the urea and ammonia mixture of the urine was markedly decreased by induced acidosis and a low protein diet, the urea clearance calculated from the excretion rate of urea alone suffered a parallel reduction. If, however, values for excretion of urea and ammonia were substituted for urea, the clearance calculated remained at the usual level. In two subjects the simultaneously performed creatinine clearances were found to have normal values. They considered these results "favor the hypothesis that the ammonia excreted in the urine of man is formed in the kidney chiefly from urea removed from the blood." Pitts (2) found that, in the dog, the urea clearance relative to the creatinine clearance was essentially the same in acidosis and alkalosis as in the normal. At low plasma urea values in acidosis the urea + ammonia clearance was considerably higher than the urea clearance, and in some instances exceeded the creatinine clearance. He concluded that if urea is the precursor of urinary ammonia, it is not the urea that has passed into the glomerular filtrate. The possibility that urea might be removed directly from the postglomerular blood and transformed to ammonia in the tubules could not be excluded.

In the present work, various physiological functions of the kidney have been studied during acidosis in dogs with only one kidney in which the kidney had previously been explanted by a modification of Rhoads' technique (3). Because explantation permits the

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simultaneous withdrawal of renal venous and femoral arterial blood, investigation can be made of the possibility of formation of urinary ammonia from postglomerular blood urea, as well as the formation of ammonia from filtered urea.

Methods

Female dogs were used in all experiments, and were prepared by the explantation of the left kidney and a subsequent right nephrectomy. They were maintained on the low protein diet of Jolliffe and Smith (4). All experiments were carried out after a fast of 18 hours or more. Acidosis was produced by feeding the dogs calcium chloride in water for several days before, and again an hour before, the experiment was begun. The total CO_2 content of the serum was found to be as low as 8.2 mM per liter in some experiments. A saline solution containing creatinine was injected intravenously throughout the experiment. The technique for explantation of the kidney, and details employed in determining clearances and in the withdrawal of blood, were the same as described by Gordon, Alving, Kretzschmar, and Alpert (5).

Urea nitrogen in urine and in whole blood was determined by the method of Van Slyke (6). Creatinine was determined in urine, in plasma, and in blood by the method of Folin and Wu (7). Urinary ammonia nitrogen was determined by the method of Van Slyke and Cullen (8). Cell and plasma volumes were determined by the Wintrobe (9) hematocrit. All analyses were carried out in duplicate, and in triplicate when blood urea determinations and creatinine determinations did not agree within 0.1 mg. per 100 cc. and within 0.5 per cent, respectively.

Calculations

Experimental and theoretical support for the validity of formulæ expressing various physiological activities of the kidneys has been presented by Møller, McIntosh, and Van Slyke (10), Van Slyke, Rhoads, Hiller, and Alving (11), and Van Slyke, Hiller, and Miller (12), and, therefore, will not be discussed. The formulæ and definitions of symbols are as follows:

Clearance (C *or* C_p)—The volume of whole blood or of plasma containing the amount of excretory substance that is eliminated per minute in the urine.

$$\text{Creatinine, } C_p = \frac{UV/A_p}{S}$$

$$\text{Urea, } C = \frac{UV/A}{S}$$

$$\text{Urea + ammonia, } C = \frac{((U + \mathrm{NH_3})V)/A}{S}$$

A or A_p = mg. excretory substance contained in 1 cc. arterial whole blood or arterial plasma
U = mg. excretory substance in 1 cc. urine
$\mathrm{NH_3}$ = mg. ammonia nitrogen in 1 cc. urine
V = cc. urine excreted per minute
S = surface area expressed in sq.m.

Extraction Percentage (*E or E_p*)—The percentage of urea or creatinine removed from the blood or plasma as it perfuses through the kidney.

$$\text{Urea (observed), } E = E_p = \frac{A - R}{A} \times 100$$

$$\text{Plasma creatinine (observed), } E_p = \frac{A_p - R_p}{A_p} \times 100$$

Calculated by assuming that all the extracted urea comes from plasma,

$$\text{Plasma urea, } E_p = E_p \text{ observed} \left(1 + 0.75 \frac{V_c}{V_p}\right)$$

R or R_p = mg. excretory substance contained in 1 cc. renal venous blood or renal venous plasma
0.75 = ratio of cell water to plasma water
V_c or V_p = volume of cells or plasma in 1 volume of blood

Urea Reabsorption—The fraction of filtered urea reabsorbed in the tubules during the 30 to 60 minute period of urea clearance determination or during the 1 to 2 minute period of blood withdrawal.

$$\text{Urea reabsorption over 30 to 60 minute period, } 1 - \frac{\text{urea } C_p}{\text{creatinine } C_p}$$

$$\text{Urea reabsorption over 1 to 2 minute period, } 1 - \frac{\text{calculated urea } E_p}{100\,\alpha}$$

α = fraction of arterial water filtered in glomeruli (100α = creatinine E_p)

TABLE I

Experimental Results

Type of determination	Determination	Dog I		Dog II		Dog III		Mean		
		No. of experiments						Values for 3 dogs		Per cent change during acidosis
		3	6	6	4	7	4			
		Control	Acidosis	Control	Acidosis	Control	Acidosis	Control	Acidosis	
Clearance	Plasma creatinine clearance, *cc. per sq. m.*	44.0	16.4	49.1	35.8	55.3	42.7	49.5	31.6	−36
	Blood urea clearance, *cc. per sq. m.*	25.8	10.3	27.4	19.1	27.7	24.0	27.0	17.8	−34
	Blood urea clearance/plasma creatinine clearance	0.59	0.63	0.56	0.53	0.51	0.58	0.53	0.56	+6
	Blood urea + ammonia clearance, *cc. per sq. m.*	27.5	13.1	29.5	24.9	28.6	29.8	28.5	22.2	−22
	Blood urea + ammonia clearance/plasma creatinine clearance	0.62	0.80	0.60	0.70	0.52	0.68	0.57	0.71	+25
Extraction and reabsorption	Creatinine extraction from plasma, %	18.3	14.3	19.1	19.6	17.9	16.1	18.5	16.7	−9
	Urea extraction from whole blood, %	7.9	6.2	8.5	9.5	6.9	5.2	7.8	7.0	−10
	Calculated plasma urea extracted, %*	12.4	8.6	12.6	13.5	7.6	7.2	10.9	9.7	−10
	Urea reabsorption; calculated from urea *vs.* creatinine clearances over 30–60 min. period, %	49	42	49	51	56	47	51	47	−9
	Urea reabsorption; calculated from urea *vs.* creatinine extractions, %	33	39	28	32	60	56	40	42	+5

Renal blood flow	Renal blood flow; calculated from creatinine, *cc. per sq. m.*	364	170	351	225	433	331	383	241	−37†
	Renal blood flow; calculated from urea, *cc. per sq. m.*	348	169	364‡	208	509	352‡	407	243	−37†
	Ratio of calculated blood flows, urea/creatinine	0.96	0.99	1.04	0.92	1.18	1.06	1.06	1.01	
	Renal blood flow; calculated from urea + ammonia, *cc. per sq. m.*	368	214	388‡	270	525	442‡	427	309	−19†
	Ratio of calculated blood flows, urea + ammonia/creatinine	1.01	1.26	1.11	1.20	1.21	1.34	1.12	1.28	

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* Calculated by assuming that all the extracted urea comes from the plasma. The difference between this value and the creatinine extraction is used to estimate the proportion of filtered urea which is reabsorbed.

† Per cent of 383 cc. the mean control value.

‡ One renal blood flow calculation is omitted because urea extraction was only 1.5 per cent of arterial blood urea content during the transient shut-down of urea excretion.

Renal Blood Flow (*F*)—The number of cc. of blood flowing through the kidney per minute.

$$\text{Creatinine or urea, } F = \frac{UV/(A - R)}{S}$$

$$\text{Urea + ammonia, } F = \frac{(U + NH_3)/(A - R)}{S}$$

Because $A - R$ is much less variable in the case of creatinine than in the case of urea (5), the blood flow calculated from creatinine values is less subject to error than blood flow calculated from urea values, and is used as the standard of reference in judging the validity of blood flow determinations in which other values are employed.

Results

The mean of individual experiments on each dog and the average for three dogs are given in Table I. Results on individual dogs were in good agreement.

Clearances—During acidosis the average plasma creatinine clearance fell 36 per cent, and the blood urea clearance, 34 per cent. In individual experiments the fall varied greatly according to the degree and duration of acidosis, but the ratio of the two clearances remained fairly constant. The average blood urea + ammonia clearance, in contrast to the urea clearance, fell only 22 per cent. The urea clearance to creatinine clearance ratio remained constant (+6 per cent) during acidosis, while the urea + ammonia clearance to creatinine clearance ratio increased 25 per cent, exceeding unity in one experiment. These results agree with those of Pitts (2).

Reabsorption of Urea—Acidosis did not affect significantly the reabsorption of urea, either as estimated from simultaneous urea and creatinine clearances or from the simultaneous extraction percentages of urea and creatinine.

Extraction Percentages—The average plasma creatinine extraction fell slightly during acidosis. The average observed whole blood urea extraction and the plasma urea extraction, estimated after allowing for diffusion of urea from cells, were similarly slightly decreased. The fall in extractions was of questionable significance.

Renal Blood Flow—During control periods when ammonia excretion was negligible, average renal blood flow estimations based on urea and urea + ammonia values were 6 per cent and 12 per cent higher, respectively, than the renal blood flow estimated from creatinine. In individual experiments the blood flow estimated from creatinine and urea values were frequently at variance because occasionally great momentary fluctuations in the reabsorption of urea make its extraction less constant than the creatinine extraction, which changes only with variations in filtration (5). The inconstancy of urea extraction invalidates comparison of renal blood flow figures in single experiments, but does not vitiate the significance of comparisons based on the averages of several experiments. During acidosis average diminution in renal blood flow, estimated either from creatinine or urea values, was of the same order of magnitude as the diminution in urea and creatinine clearances. Both blood flow estimations were 37 per cent below the control (creatinine) blood flow measured during periods without acidosis. The average blood flow during acidosis estimated by calculating urinary ammonia as urea was only 19 per cent lower than the (creatinine) control, and was 28 per cent higher than the blood flow calculated either from creatinine or urea values during acidosis.

DISCUSSION

As premises for the following discussion it will be assumed, as appears probable (11, 12, 5), that normally approximately 20 per cent of the water and filtrable solutes of the plasma, including creatinine and urea, is filtered in the glomeruli, and that, of the filtered urea, on the average about 40 to 50 per cent is reabsorbed. No creatinine appears to be reabsorbed. (A summary of values expected on the basis of the theoretical considerations discussed below, compared to average values obtained experimentally, is given in Table II.)

In the dog, the *urea clearance* calculated from the excretion rate of urea alone has the same ratio to the creatinine clearance during acidosis that it has under normal conditions. Therefore, if the creatinine clearance represents the glomerular filtrate, the urea clearance is proportional to that filtrate. When, on the other hand, the value for the excretion of urea + ammonia is substituted for urea, the ratio of the clearance thus calculated to the creatinine

TABLE II

Values Expected from Theoretical Considerations Compared with Values Found Experimentally

For explanation of formulæ, see text.

Physiological functions studied		Theoretical values to be expected during acidosis			Experimental values found during acidosis
		If urinary NH_3 is formed in kidney from		If urinary NH_3 is not formed in kidney from urea	
		Filtered urea	Reabsorbed or postglomerular urea		
Blood urea clearance					
Urea.........	$\frac{UV}{A}$	Low*	Correct	Correct	Correct (+6%)
" + NH_3.........	$\frac{(U + NH_3)V}{A}$	Correct†	High‡	High‡	High (+25%)
Urea extraction percentage...	$\frac{A - R}{A} \times 100$	Normal	" §	Normal	Normal ? (−10%)
" reabsorption (momentary)............	$1 - \frac{\text{calculated urea } E_p}{\text{creatinine } E_p}$	"	Low‖	"	Normal (+5%)
Renal blood flow					
Urea.........	$\frac{UV}{A - R}$	Low*	" §	Correct	Correct (+1%)
" + NH_3.........	$\frac{(U + NH_3)V}{A - R}$	Correct	Correct¶	High‡	High (+28%)

* Numerator too low.

† Creatinine clearance and blood flow values are used as standards of reference in determining "correct" urea clearance and blood flow values.

‡ Numerator too high.

§ R too low.

‖ Urea E_p too high (see §).

¶ Numerator and denominator increased proportionately.

clearance increases with increased ammonia output. These results, in agreement with those found by Pitts (2) in experiments on the dog, render improbable the formation of urinary ammonia in significant amounts from urea that has passed into the glomerular filtrate. The possibility of formation of urinary ammonia from reabsorbed or postglomerular urea,[1] however, cannot be excluded. These results with dogs are opposite to those observed in man by Van Slyke, Page, Hiller, and Kirk (1), who found that the ratio, ammonia + urea clearance to creatinine clearance, rather than the ratio, urea clearance to creatinine clearance, remained unaffected by acidosis and high ammonia formation in human subjects.

The *percentage of urea extracted* from the arterial blood during passage through the kidney is, like the extraction of creatinine, only slightly diminished, or normal during acidosis.

Urea reabsorption, calculated from either the clearances or the extraction ratios of urea and creatinine, is normal during acidosis. The approximate constancy of the ratio, creatinine extraction to urea extraction, would be expected during increased ammonia production only if (*a*) the ammonia were formed from some precursor other than urea, or (*b*) the ammonia were formed from the fraction of filtered urea that is not reabsorbed, or (*c*) the ammonia were formed from reabsorbed or postglomerular urea, and the reabsorption of urea were increased exactly enough to balance the formed and excreted ammonia. Of these possibilities (*a*) appears compatible with all the observed facts, (*b*) does not appear probable for reasons advanced previously in the discussion of clearance ratios, and (*c*) would appear improbable because increased urea reabsorption was not observed. The normal extraction percentages and normal estimated urea reabsorption, therefore, add to the weight of evidence against urea as the source of ammonia in the kidney of the dog.

During both control periods and periods of acidosis, estimations of *renal blood flow* based on the excretion rate of urea alone are the same as those based on creatinine, but those based on the excretion of urea + ammonia are higher (significantly so during acidosis). This fact, for reasons similar to those given above, also weighs against ammonia formation from urea.

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[1] By postglomerular urea is meant the urea that has passed through the glomerulus without being filtered.

When a fall in the urea or creatinine clearance occurred during acidosis, it was accompanied in our experiments by a diminution in renal blood flow, and only to an insignificant extent, if at all, by a diminution of the percentages of these substances extracted from the arterial blood during passage through the kidney. The parallelism between the urea and creatinine clearance changes and renal blood flow changes, observed in dogs by previous authors (11, 12) holds equally well when ammonia output is increased by $CaCl_2$ acidosis.

SUMMARY

The ammonia to urea ratio in the urine of dogs with kidneys explanted according to Rhoads' procedure was increased by a combination of low protein diet and $CaCl_2$ acidosis, and the results were compared with those observed in the same animals on low protein diets, with relatively slight ammonia outputs.

The creatinine to urea ratio, with regard to both the percentages extracted from the blood plasma during passage through the kidney, and with regard to the clearances of the two substances, remained the same during acidosis as before. Also the blood flows, calculated from the extraction and excretion rates of the two substances, were alike.

On the other hand, if urea + ammonia was substituted for urinary urea in calculating the urea clearance, the ratio of the clearance thus calculated to the creatinine clearance was increased by the acidosis; the blood flow calculated by similar substitution also became greater than that estimated from creatinine.

These results appear contrary to the probability that in dogs urea is the source of the urinary ammonia formed in the kidneys.

BIBLIOGRAPHY

1. Van Slyke, D. D., Page, I. H., Hiller, A., and Kirk, E., *J. Clin. Inv.*, **14,** 901 (1935).
2. Pitts, R. F., *J. Clin. Inv.*, **15,** 571 (1936).
3. Rhoads, C. P., *Am. J. Physiol.*, **109,** 324 (1934).
4. Jolliffe, N., and Smith, H. W., *Am. J. Physiol.*, **99,** 101 (1931).
5. Gordon, W., Alving, A. S., Kretzschmar, N. R., and Alpert, L., *Am. J. Physiol.*, **119,** 483 (1937).
6. Van Slyke, D. D., *J. Biol. Chem.*, **73,** 695 (1927).
7. Folin, O., and Wu, H., *J. Biol. Chem.*, **38,** 81 (1919).

8. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 211 (1914); **24**, 117 (1916).
9. Wintrobe, M. M., *J. Lab. and Clin. Med.*, **15**, 287 (1929–30).
10. Møller, E., McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Inv.*, **6**, 427 (1928).
11. Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A. S., *Am. J. Physiol.*, **109**, 336 (1934).
12. Van Slyke, D. D., Hiller, A., and Miller, B. F., *Am. J. Physiol.*, **113**, 611 (1935); **113**, 629 (1935).

PORPHYRIN EXCRETION IN THE FECES IN NORMAL AND PATHOLOGICAL CONDITIONS

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The problem of the relation of porphyrin metabolism to the normal and faulty construction and destruction of the respiratory pigments has been the subject of a number of communications. More data based upon more satisfactory analytical methods are required, however, before adequate conclusions can be drawn. Such data should concern the kind and type of porphyrin excreted in normal and pathological states.

In a previous communication we have discussed the excretion of urinary porphyrins in disease (1). This communication describes the methods of determining porphyrins in the feces and the results which have been obtained from the determinations.

Methods

In principle the methods used are similar to those previously described (1) and are based on the work of Fischer and his school (2). Unlike urine, feces contain three or more different kinds of porphyrins. A clinical method was devised for the rapid and accurate separation and isolation of these various porphyrins in a pure state. In order to identify properly the porphyrins a high degree of purity is indispensable. The methods of separation, purification, and identification are summarized in Tables I and II and are described in detail below.

It is essential that all the reagents used be free from copper and zinc, for contamination with these metals leads to the formation of metallic porphyrin compounds. Peroxide-free ether should be used in all instances.

I. Preparation of Feces and Extraction of Porphyrins

a. Formed Feces—The feces passed in 3 day periods are collected in dark glass bottles or in enameled containers. A small amount of toluene is added, and the material is stored in an ice box until the actual porphyrin extraction is made.

To the feces are added 3 to 5 times their volume of glacial acetic acid and the mixture is ground with an electric stirrer to a homogeneous paste. 10 cc. of a saturated solution of sodium acetate are then added for each 300.0 cc. of mixture.

Table I

Scheme for Separation of Fecal Porphyrins

<table>
<tr><td colspan="3">I. Extraction of the total crude porphyrins with ether. Purification of total porphyrins</td></tr>
<tr><td colspan="3">II-A. Saponification of the natural porphyrin esters with 20% NaOH. Separation of soluble and insoluble sodium salts</td></tr>
<tr><td rowspan="3">II-<i>a</i>. Soluble sodium salts, <i>coproporphyrins</i></td><td colspan="2">II-<i>b</i>. Insoluble sodium salts, <i>deuteroporphyrin</i> and <i>protoporphyrin</i></td></tr>
<tr><td colspan="2">II-B. Separation of deutero- and protoporphyrins with 0.6% HCl from ether</td></tr>
<tr><td>0.6% HCl fraction, deuteroporphyrin (III-B)</td><td>Ether fraction, protoporphyrin (III-C)</td></tr>
</table>

The fecal suspension in portions of 300 to 500 cc. is then extracted 3 to 5 times, with 1000 to 1500 cc. of ether for each extraction. Smaller ether volumes may lead to the formation of inseparable emulsions. In the event of an emulsion, separation may be attained by the addition of large volumes of ether and glacial acetic acid. After the third ether extraction about 50.0 cc. of glacial acetic acid are added before further extractions are attempted. The first three extracts contain practically all the coproporphyrins; further extraction may, however, be necessary for the complete removal of protoporphyrin.

The combined ether extracts are washed with one-fifth of their volume of dilute sodium acetate solution (1 per cent) until the wash water is practically colorless. The washed ether is extracted three times with 5 per cent HCl, 50 cc. of HCl being used for each 1500 cc. of ether. Occasionally porphyrins are retained in the ether, owing to the presence of colloids, in which case further extraction is carried out with 10 to 20 per cent HCl. The combined HCl extracts are brought to a negative reaction to Congo red by the addition of solid sodium acetate, one-tenth of the volume

TABLE II

Scheme for Purification of Fecal Porphyrins

<table>
<tr><th colspan="2">III-A. <i>Coproporphyrins</i></th><th>III-B. <i>Deuteroporphyrin</i></th><th>III-C. <i>Protoporphyrin</i></th></tr>
<tr><td colspan="2">Extracted from ether with 0.2% HCl</td><td>Extracted from ether with 0.6% HCl</td><td>Remains in ether after extraction with 1.0% HCl</td></tr>
<tr><td colspan="2">Purification with chloroform and petroleum ether</td><td>HCl concentration reduced to 0.2% and extracted with chloroform</td><td>Extracted with 5% HCl and dissolved in chloroform</td></tr>
<tr><td colspan="2">Coproporphyrins in HCl</td><td>Deuteroporphyrin in chloroform</td><td>Protoporphyrin in chloroform</td></tr>
<tr><td colspan="4">IV. Identification</td></tr>
<tr><td colspan="2">As coproporphyrin methyl esters</td><td>As deuteroporphyrin methyl esters</td><td>As protoporphyrin or mesoporphyrin methyl esters</td></tr>
<tr><td colspan="2">V. Separation of coproporphyrin I and III methyl esters with methyl alcohol</td><td></td><td></td></tr>
<tr><td colspan="2">Methyl alcohol</td><td></td><td></td></tr>
<tr><td>Soluble</td><td>Insoluble</td><td></td><td></td></tr>
<tr><td>Coproporphyrin methyl ester III</td><td>Coproporphyrin methyl ester I</td><td></td><td></td></tr>
</table>

of glacial acetic acid is added, and the porphyrins are then extracted three times with fresh ether. The combined ethers are washed several times with diluted sodium acetate and the porphyrins are reextracted with HCl.[1] This procedure of driving the porphyrins back and forth between ether and 5 per cent HCl is repeated several times until practically no bile pigments remain. If the ether-HCl separation proceeds slowly, the separation of the layers can be markedly accelerated by the addition of between 10.0 and 50.0 cc. of glacial acetic acid. The completeness of extraction is controlled in all instances by spectroscopic examination or by the absence of fluorescence in ultraviolet light.

During the process large amounts of bile pigments are removed and the ether and HCl volumes progressively reduced. Substances precipitating at the interface, such as copronigrin, greenish and brownish pigments, are filtered off and then are dissolved in a few cc. of concentrated HCl, so that precipitating porphyrins are not overlooked. The final combined HCl extracts are extracted twice with petroleum ether, thereby removing lipids and cholesterols.

b. Semiliquid and Liquid Feces (Enemas)—200 to 250 cc. of glacial acetic acid and 10.0 cc. of saturated and sodium acetate are added for each 500 cc. of fecal solution. Solid fecal particles are broken up, and the homogeneous mixture is then extracted in 300 to 500 cc. portions with 3 to 5 times its volume of ether. The ether extracts are combined with those of (*a*) or purified according to (*a*).

II. Separation of the Porphyrins

Separation of the porphyrins can be accomplished successfully both quantitatively and qualitatively only if the procedures under (I) have been carefully followed and practically all bile pigments have been removed.

Esters of porphyrins are to be expected (3), and difficulties in porphyrin separation will ensue if they are not first saponified.

In principle the separation of the porphyrins is similar to that described for the urinary porphyrins in an earlier paper (1), different HCl concentrations (HCl numbers), the solubility of the porphyrins in chloroform, and the solubility of the sodium salts of the porphyrins in 20 per cent NaOH being utilized in the separation process (Table III).

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[1] Each time the porphyrins are driven into ether from an HCl solution the procedure described must be used.

II-A. Saponification of Porphyrin Esters; Separation of Porphyrins into Soluble and Insoluble Sodium Salts—Following the petroleum ether extraction the porphyrins are driven from the HCl into ether[1] and are then extracted from ether with 20 to 50 cc. of 20 per cent NaOH. Insoluble sodium salts of the porphyrins precipitate at the interface of the ether and NaOH solutions; soluble porphyrins remain in the NaOH layer. After the solutions have stood overnight, the soluble and insoluble porphyrins are separated by filtration. The precipitate is washed with small amounts of 20 per cent NaOH. The separation is practically quantitative. The filtrate (II-*a*) contains the coproporphyrins. The precipitate (II-*b*) contains the deutero- and protoporphyrin.

TABLE III

Physical Constants of Important Porphyrins (*4*)

	HCl No.*		Melting points of methyl ester	Solubility of free porphyrin in NaOH
	Free porphyrin	Methyl ester		
			°C.	
Protoporphyrin IX......	2.0	5.5	228	Insoluble
Mesoporphyrin IX	0.5	2.5	216	"
Deuteroporphyrin IX .	0.4	2.0	223	"
Hematoporphyrin IX	0.1		212	"
Coproporphyrin I......	0.08	1.5	252	Soluble
" III......	0.09	1.5	142† 172	"
Uroporphyrin I......		7 *Ca.*	302	"
" III		7 "	256	"

* The concentration of hydrochloric acid which will remove two-thirds of the porphyrin from an ether solution of the porphyrin when equal volumes are shaken together.

† Double melting point.

II-B. Separation of Deutero- and Protoporphyrins—The precipitate (II-B) containing deutero- and protoporphyrins is dissolved in glacial acetic acid or in 20 per cent HCl and is then driven into ether. The ether is washed and extracted several times with 0.2

per cent HCl in order to remove any remaining coproporphyrin and biliviolin. If precipitates form, they are filtered off. The purified ether is then extracted several times with one-third its volume of 0.6 per cent HCl. The HCl extract (III-B) contains the deuteroporphyrin. The remaining ether (III-C) contains the protoporphyrin.[2]

III. Purification of the Porphyrins

III-A. Final Purification of Coproporphyrins—The filtrate (III-A) containing the coproporphyrins is acidified with glacial acetic acid and allowed to cool. The porphyrins are extracted with ether and the ether is washed several times with dilute sodium acetate solution. The coproporphyrins are completely removed by repeated extractions with 0.2 per cent HCl. The hydrochloric acid solution is then extracted 3 to 5 times with one-third its volume of alcohol-free chloroform and twice with petroleum ether. If the porphyrin concentration is too high, coproporphyrin may precipitate out and must be recovered by filtration and purified as stated above. The HCl solution is then filtered and the coproporphyrin driven into ether. The ether, containing the pure coproporphyrin, is washed, filtered through a double paper filter, evaporated in a distilling flask, and the porphyrins are esterified.

III-B. Final Purification of Deuteroporphyrin—The porphyrins of the HCl extract (III-B) are driven into ether, washed, and extracted with 0.2 per cent HCl, in order to remove traces of coproporphyrins, and then extracted with 0.6 per cent HCl.

This solution containing all the deuteroporphyrin is extracted several times with alcohol-free chloroform, so that traces of protoporphyrin are removed. The 0.6 per cent HCl solution is then diluted to 0.2 per cent HCl, and the deuteroporphyrin removed by repeated chloroform extraction. The chloroform is washed with distilled water, filtered through a double paper filter, evaporated in a distilling flask, and the porphyrin esterified.

III-C. Final Purification of Protoporphyrin and Reduction to Mesoporphyrin—The ether (III-C) containing the protoporphyrin is extracted with 1.5 per cent HCl in order to remove any remaining pigments and decomposition products of protoporphyrin.[3] Further purification is attained by extracting the protoporphyrin from the ether with 3 to 5 per cent HCl. The porphyrins are then extracted from the HCl solution with chloroform, the chloroform washed, filtered through a double paper filter, evaporated in a distilling flask, and the porphyrins and esterified.

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[2] Although hematoporphyrin and mesoporphyrin have not been found as physiological or pathological products, if present both should be found in III-B.

Identification as the methyl ester of protoporphyrin is difficult, since good crystallization is seldom obtained. Somewhat better results can be obtained by identification as Cu salts of the methyl ester. Identification is best accomplished, however, by reduction of the protoporphyrin to mesoporphyrin and identification as mesoporphyrin ester, which crystallizes with ease. Reduction was carried out according to the method devised by Fischer (5). Lemberg's modification of the Fischer method was found to be of value for final purification of the mesoporphyrin after reduction (6).

IV. Esterification, Purification, and Identification of Porphyrin Esters

The porphyrins are esterified with absolute methyl alcohol-HCl, as described in a previous publication (1). The following procedures are helpful adjuncts for further purification. After chloroform purification the esters are dried and then washed with boiling petroleum ether. The esters are then dissolved in 1 to 3 cc. of chloroform, to which 10 times the amount of ether, containing a few drops of diluted acetic acid, are added. The mixture is then washed with distilled water and extracted with HCl, in a concentration equivalent to two-thirds the concentration of the acid number of the particular porphyrin ester involved. Traces of unesterified porphyrins are thus removed. The brown pigments which precipitate out are filtered off. The purified ester in ether is carefully washed with distilled water, filtered through a double paper filter, and evaporated. Crystallization takes place from a 1:1 hot mixture of absolute methyl alcohol and chloroform. Pure crystals are usually obtained after the second recrystallization.

Final identification is accomplished, by melting point determination, and comparison of the absorption spectra with those of known porphyrin methyl esters. Identification by crystal forms is not reliable, since porphyrin esters do not always form specific crystals.

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[3] Since protoporphyrin with two vinyl groups is a labile complex, it is partially changed in the intestinal tract and by the procedures used for separation.

V. Separation of Porphyrin Isomers

V-A. Separation of Coproporphyrin I and III (*3*)—Advantage is taken of the fact that the methyl ester of coproporphyrin I is practically insoluble in cold absolute methyl alcohol, while that of coproporphyrin III is extremely soluble (6). Separation is attempted only after complete purification of the esters (IV). Two fractions are obtained: (*a*) methyl alcohol-insoluble fraction; coproporphyrin I (After one or two recrystallizations pure coproporphyrin I methyl ester is easily obtained. After each crystallization the crystals are washed with methyl alcohol); (*b*) methyl alcohol-soluble fraction; coproporphyrin III.

Owing to the presence of small amounts of brownish pigments, traces of coproporphyrin I methyl ester remain in the methyl alcohol-soluble fraction. Difficulty is usually encountered in removing the traces of brownish pigment and coproporphyrin I ester. If sufficient ester is present, repeated recrystallizations from methyl alcohol chloroform yield a pure product. The methyl alcohol-insoluble fraction is discarded after each recrystallization. In some instances good results were obtained by dissolving the ester in very small amounts of chloroform, to which 10 to 30 times the amount of petroleum ether are added. The amount of ether added should be small enough to prevent instant precipitation of the porphyrins. The brownish pigments usually precipitate out and they can be filtered off; the filtrate is then evaporated to dryness, dissolved in methyl alcohol, and recrystallized. In most instances after the above procedures no alcohol-soluble fraction remained, and the porphyrin was found to be coproporphyrin I ester, rendered soluble by the contaminating traces of brownish pigments.

Detection and Separation of Unknown Porphyrins—In certain pathological conditions porphyrins other than copro-, deutero-, and protoporphyrins were found, and are to be expected (1, 7). These can only be detected by superimposing the spectrum of the separated porphyrins against that of known porphyrins. Unknown porphyrins are isolated by the use of serial HCl solutions, the concentrations of which should not differ by more than 0.1 to 0.2 per cent, and by the solubility in NaOH of different concentration, as well as the solubility in chloroform.

Detection and Separation of Natural Porphyrin Esters—Physiologically excreted coproporphyrin esters can only be detected if the total crude porphyrins are not saponified. They can be extracted from ether solutions of the total crude porphyrins by the use of 0.3 to 0.7 per cent HCl. They can then easily be removed by extraction with chloroform. Details of this method will be discussed in a later publication.

TABLE IV

Fecal Excretion of Coproporphyrin I

Clinical diagnosis	Case No.	Fecal collections; duration	Average daily fecal excretion	Average daily total excretion (urinary and fecal)	Porphyrin methyl ester; m.p.
		days	*micrograms*	*micrograms*	*°C.*
Normal	1	9	231	318	228
"	2	9	205	328	234
"	3	9	274	376	241
"	4	9	241	362	236
"	5	9	242	306	
Hemolytic jaundice	6-*a*	10	401	719	244
	6-*b*	6	290	371	
" "	7	14			237

Results

The results are presented in Tables IV and V. The quantitative coproporphyrin values were obtained by measurements with a photoelectric colorimeter. Quantitative urinary and fecal porphyrin data are listed only for the normals and for one case of hemolytic jaundice.

The melting points of the natural coproporphyrin methyl esters are in all cases lower than those of the synthetic esters. The factors responsible for this difference are discussed in a previous paper on urinary porphyrins (1). Only those melting points are recorded in which adequate crystallization was obtained.

In Case 6 (Table IV), hemolytic jaundice, the quantitative values (*a*) are those obtained before splenectomy and the values (*b*) are the quantitative values after splenectomy.

Special care was taken in the isolation of isomeric compounds and in several instances it was possible to raise the melting points of the coproporphyrin esters in the alcohol-insoluble fraction 10–20°. In the case of pellagra, there was evidence suggesting excretion of coproporphyrin III as well as coproporphyrin I; however, final proof for coproporphyrin III is lacking. In the case of lead poisoning, the simultaneous excretion of coproporphyrins I and III is demonstrated. In the instance of pigment cirrhosis of the liver, only coproporphyrin III could be isolated, although the presence of small amounts of coproporphyrin I was suspected. In all the other cases only coproporphyrin I could be isolated. Differences in crystal forms are of little value for final identification of the coproporphyrin esters. The ease of crystallization is a more helpful index.

TABLE V

Fecal Excretion of Coproporphyrins

	Clinical diagnosis	No. of cases	Melting points
			°*C.*
Coproporphyrin I	Pernicious anemia	4	236, 238, 238, 235
	Polycythemia vera	1	238
	Lobar pneumonia	3	240, 245, 234
	Atrophic cirrhosis of liver	4	243, 238, 241, 232
	Catarrhal jaundice	2	230, 234
	Obstructive jaundice	2	232, 234
	Addison's disease	1	239
	Hodgkin's "	1	237
	Pellagra	1	234
Coproporphyrins I and III	Lead poisoning	1	228 (I), 138, 166 (III)
Coproporphyrin III	Pigment cirrhosis of liver	1	136, 166

The results of the protoporphyrin and deuteroporphyrin determinations are reserved for a later publication. In some cases on a meat-free diet and in which gastrointestinal hemorrhage could be excluded, relatively large amounts of deuteroporphyrin and protoporphyrin were encountered. Meso- and hematoporphyrins were not detected in any of the cases studied.

Very small amounts of a special kind of chloroform-soluble porphyrin were encountered in most instances. Spectroscopically it is identical with coproporphyrin and after saponification it is no longer chloroform-soluble, and exhibits all the physical properties of coproporphyrin. Sufficient material was not available to permit determination of the esterifying group of this natural ester.

DISCUSSION

As shown in Tables IV and V coproporphyrin I is the porphyrin excreted in the feces in normal and most pathological states. These results are similar to those previously reported for the urinary porphyrins (1) and they extend and confirm the results recorded in the literature (2, 8).

The rate of urinary and fecal excretion of coproporphyrin I is dependent on many factors, most important of which are the production rate and the liver function.

Although the simultaneous production of Type I and Type III porphyrins, called the dualism of the porphyrins, has been definitely established in normals and in disease, a satisfactory explanation of this phenomenon is lacking. All of the known respiratory pigments are Type III derivatives (hemoglobin, myoglobin, cytochrome C, and catalase) (6, 9–11). Type I porphyrins cannot be derived by degradation of these Type III compounds. Type I respiratory pigments have not been demonstrated as yet. Type I porphyrins must be derived by a special synthesis from simple pyrrole compounds (12).

Quantitative studies show that in normal adults with a constant hematopoietic activity as shown by the Type III porphyrin production (hemoglobin) the total coproporphyrin I excretion falls within the same range in all instances. Diseases which are characterized physiologically by increased hematopoietic activity show an increased excretion of coproporphyrin I. This is the case, for example, in hemolytic jaundice, a condition marked by a great increase of blood production. Following splenectomy in hemolytic jaundice both the increased hematopoietic activity and the excretion of coproporphyrin I revert toward normal. These observations suggest strongly the existence of a directly proportional relationship between the synthesis of Type III and Type I porphyrins; in other words, a directly proportional relationship exists between hematopoietic activity and production rate of coproporphyrin I.

In certain pathological states with faulty porphyrin metabolism, especially in the group of porphyria, this direct proportion is disturbed (13).

The fecal excretion of coproporphyrin III has been doubted in the past. It was shown to exist in chronic porphyria (3, 14) and here it has been demonstrated in one case of liver cirrhosis and in lead poisoning. Two of the above cases showed simultaneous excretion of coproporphyrins I and III. This phenomenon warrants further careful observation.

SUMMARY

Coproporphyrin I is excreted in the feces in normals and most pathological states. Coproporphyrin III was found in one case of liver cirrhosis. Simultaneous excretion of coproporphyrin III and small amounts of coproporphyrin I were encountered in lead poisoning. In normal individuals and in certain disease states coproporphyrin I production and hematopoietic activity (production of Type III porphyrin compounds) appear to be directly proportional.

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BIBLIOGRAPHY

1. Dobriner, K., *J. Biol. Chem.*, **113**, 1 (1936).
2. Fischer, H., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin, Abt. I, Teil 11, 169 (1926). Fischer, H., and Duesberg, R., *Arch. exp. Path. u. Pharmakol.*, **166**, 95 (1932). Watson, C. J., *Z. physiol. Chem.*, **204**, 57 (1932); **208**, 101 (1932); *J. Clin. Inv.*, **14**, 110 (1935).
3. Dobriner, K., *Proc. Soc. Exp. Biol. and Med.*, **35**, 175 (1936).

4. Fischer, H., and Treibs, A., in Oppenheimer, C., and Pincussen, L., Tabulæ biologicæ, Berlin, **3**, 339 (1926). Kirstahler, A., in Oppenheimer, C., and Pincussen, L., Tabulæ biologicæ periodicæ, Berlin, **1**, 48 (1931). Fischer, H., and Libowitz, H., *Z. physiol. Chem.*, **241**, 220 (1936). Fischer, H., and Hofmann, H. J., *Z. physiol. Chem.*, **246**, 15 (1937).
5. Fischer, H., and Puetzer, B., *Z. physiol. Chem.*, **154**, 47 (1926). Fischer, H., and Jordan, E., *Z. physiol. Chem.*, **190**, 91 (1930). Fischer, H., Treibs, A., and Zeile, K., *Z. physiol. Chem.*, **193**, 150 (1931).
6. Stern, K. G., *J. Biol. Chem.*, **112**, 661 (1935–36).
7. Watson, C. J., *J. Clin. Inv.*, **14**, 110 (1935).
8. Papendieck, A., *Z. physiol. Chem.*, **128**, 117 (1923). Fischer, H., and Schneller, K., *Z. physiol. Chem.*, **130**, 306 (1923); **135**, 290 (1924).
9. Fischer, H., and Lindner, F., *Z. physiol. Chem.*, **145**, 202 (1925). Fischer, H., and Stangler, G., *Ann. Chem.*, **459**, 53 (1928).
10. Schönheimer, R., *Z. physiol. Chem.*, **180**, 144 (1929).
11. Zeile, K., Reuter, F., *Z. physiol. Chem.*, **221**, 101 (1933).
12. Fischer, H., *Ber. chem. Ges.*, **60**, 2611 (1927); *Verhandl. deutsch. Ges. inn. Med.*, **45**, 7 (1933).
13. Dobriner, K., Localio, S., and Strain, W. H., *Proc. Am. Soc. Biol. Chem.*, **8**, xxvi (1936) (*J. Biol. Chem.*, **114** (1936)).
14. van den Bergh, H., *Arch. Verdauungskr.*, **42**, 306 (1928).

TISSUE RESPIRATION STUDIES ON NORMAL AND SCORBUTIC GUINEA PIG LIVER AND KIDNEY*

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The view that vitamin C can function as a hydrogen transfer agent in tissue reactions has been widely accepted, apparently because of (*a*) its great sensitivity to reversible oxidation, (*b*) its occurrence in practically all actively respiring tissues of plants and animals of higher organization than unicellular types, and (*c*) a certain amount of evidence that the vitamin can act directly upon recognized metabolites and enzymes *in vitro* (1).

When the oxygen consumption of tissues is lowered by a deficiency of a given suspected respiratory catalyst, it is often assumed *a priori* that the substance plays a catalytic rôle. Such a conclusion may not be justified, however, because of the complexity of reactions involved in the respiratory processes. Harrison (2) has supplied the first evidence of this kind concerning the rôle of ascorbic acid. He concluded that the respiration of liver slices and minced muscle from scorbutic animals was below normal. This conclusion was drawn from oxygen consumption rates which varied from −62 to +56 per cent of the average for scorbutic liver (Q_{O_2} = 0.71, wet basis) and from −23 to +35 per cent of the average for normal liver (Q_{O_2} = 1.07, wet basis). He also found that small amounts of added ascorbic acid accelerated the respiration of scorbutic liver and muscle, but had little or no effect upon normal tissue. In these experiments Harrison "balanced" the error due to oxidation of the vitamin by having an equal amount

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of ascorbic acid, without tissue, in the left-hand vessel of the Barcroft apparatus. That this is a false "balance" has been shown by several investigators, including Quastel and Wheatley (3), who found that tissue protects the vitamin against extensive aerobic oxidation, whereas the usual buffers (Cu-contaminated) permit rapid destruction.

The latter workers considered this error "only a minor criticism of Harrison's results" and arrived at the following conclusions from their own work: (*a*) The most significant effect of ascorbic acid on rat liver respiration was the maintenance of respiration at a relatively high level. This effect was less noticeable, however, with guinea pig liver, which displayed a more constant Q_{O_2}. (*b*) The Q_{O_2} of normal guinea pig liver slices in the presence of crotonate or butyrate was increased slightly and irregularly by ascorbic acid, but "the amount of rise is only about that to be expected by the oxidation of part of the ascorbic acid added." (*c*) The Q_{O_2} of scorbutic liver in the presence of crotonate or butyrate showed greater rises than those found with normal liver, but "increases of the same order have been found occasionally with normal and apparently healthy guinea-pigs."

Phillips, Stare, and Elvehjem (4) have reported that scurvy produces "relatively little effect" on the rate of oxygen uptake of guinea pig liver. Although this conclusion is in disagreement with Harrison's findings (2), the experiments in the two laboratories were not strictly comparable. Phillips *et al.* used minced tissue in an atmosphere of air. They also concluded that, "There appears to be a slight tendency [of ascorbic acid] to increase the respiration of the liver from the scorbutic guinea pigs and not to affect or to decrease slightly the respiration of the liver from the normal guinea pigs. The data presented are insufficient from which to draw definite conclusions."

During the writing of this paper it was reported by Kato (5) that scurvy in guinea pigs produced a marked increase in the oxygen consumption rate of liver and adrenals, but little or no change in several other tissues.

It is evident from the above review of the literature that information concerning the effect of scurvy on tissue respiration is very incomplete and in marked disagreement. The present study includes the following measurements on normal and scorbutic liver and kidney: (*a*) the oxygen consumption by Warburg's direct method, (*b*) the effect of added vitamin on the oxygen consumption, (*c*) the oxygen consumption and carbon dioxide production by Warburg's indirect method, and (*d*) the anaerobic glycolysis. In addition, the oxygen consumption, carbon dioxide production, aerobic glycolysis, and R.Q. of normal and scorbutic liver have been determined by the second method of Dickens and Simer (6).

EXPERIMENTAL

Guinea pigs weighing 200 to 300 gm. were fed the standard Sherman basal ration (7) supplemented with spinach for the 1st week and with 2 mg. of crystalline vitamin C daily for at least 2 weeks thereafter. The normal animals used in the experiments weighed approximately 400 gm. Scurvy was produced in 16 to 25 days after removal of the supplementary ascorbic acid, and the animals were used at various stages of deficiency.

Approximately 15 hours before the tissues were removed for respiration studies the animals were given 1.75 gm. of glucose per kilo and fasted. The animals were killed by a blow over the occiput and the blood was drained from the internal organs by decapitation. The liver and kidneys were quickly removed and placed in warm Ringer-phosphate solution containing 0.2 per cent glucose. The same lobe (largest) of the liver was used in all experiments. Cross-section slices of liver were made and in the case of kidney care was taken to use only cortical tissue. The slices were suspended in Ringer's solution and selected for proper thickness (approximately 0.3 mm.). They were also trimmed so that they remained intact during the shaking of the flasks. Wet weights were determined by a torsion balance and dry weights at the end of the experiment by drying at 105° for at least 4 hours. The time elapsing between death of the animals and the start of the measurements was approximately 30 minutes.

Oxygen Consumption

The weighed tissues (approximately 80 mg. of liver and 40 mg. of kidney) were placed in Warburg flasks containing 3 ml. of Ringer-phosphate-glucose solution in the main vessel, and 0.3 ml. of 20 per cent KOH (+ filter paper roll) in the center cup. The flasks were filled with oxygen and after temperature equilibration, readings were taken every 15 minutes for 2 hours. The rate of shaking was 90 times per minute, above which no change of respiration rate was observed either with liver or kidney. The calculated oxygen consumptions were plotted against time, and the expressed rate of respiration was determined from the straight line thus obtained. With either liver or kidney there were very few cases in which a decreased rate of respiration could be observed within the experimental period. Determinations were made in duplicate and both results were discarded if they did not agree within 6 per cent.

Table I

Oxygen Consumption of Normal and Scorbutic Liver and Kidney

Phosphate-Ringer's solution, pH 7.4, O_2 atmosphere.

Q_{O_2}, c.mm. O_2 per mg. dry tissue per hr.

	Liver		Kidney	
	Normal	Scorbutic	Normal	Scorbutic
	3.65(1)*	4.7-5.4(3)	11.7-12.3(3)	10.7-11.7(4)
	3.8-4.2(12)	5.5-5.9(10)	12.4-13.6(13)	11.8-14.0(17)
	4.3-4.35(3)	6.0-6.4(2)	13.7-14.4(4)	14.1-14.8(4)
Average..........	4.04(16)	5.70(15)	13.0(20)	12.7(25)
" solids, %	17.0	16.7	13.6	13.7

* The number of animals falling in each group is indicated in parentheses.

To save space the individual figures are not given, but in order to illustrate the agreement between animals, the numbers of animals falling within certain limits are recorded. The per cent solids calculated as the ratio of dry weight to wet weight is likewise given.

The data in Table I indicate that there was a significant increase in the oxygen consumption of liver due to scurvy, but in kidney tissue there was no real change. The observed effect could not be due to a different water content of the scorbutic tissues, since the per cent solids remained practically constant.

Effect of Added Vitamin

The effect of *in vitro* addition of vitamin C (0.1 and 0.4 mg. per ml.) on the respiration of normal and scorbutic liver and kidney was studied. The resulting respiration was compared with that of similar slices from the same animal. The chief difficulty of interpretation in such experiments is due to the uncertainty of the increased oxygen consumption resulting from oxidation of the vitamin. Although the tissues afforded a marked protection of ascorbic acid against oxidation, the remaining oxidation could not be disregarded. This became particularly evident from the following experiments, in which the oxygen consumption of Vitamin-Ringer's solutions was compared with direct titration of the vitamin with indophenol.

A series of Warburg flasks contained 1.1 mg. of vitamin in 3 ml. of vitamin-Ringer's solution at pH 7.4. After equilibration of the flasks one was used to determine the amount of vitamin that had disappeared up to the true starting time (approximately 5 per cent). After recording the manometer readings at various intervals, successive flasks were removed for indophenol titration (8) of the remaining reduced vitamin. By this means the actual loss of vitamin could be compared with oxygen consumption during the same period. Typical data of this kind are shown in Table II.

TABLE II

Comparison of O_2 Consumption and Vitamin Loss in Ringer's Solution at pH 7.4

Total vitamin 1.10 mg., $T = 37°$, alkali in center cup.

Time	O_2 calculated from titration figures	O_2 observed
	c.mm.	*c.mm.*
15	8.5	11.3
25	14 2	22 6
35	18 8	30 4
50	25.6	44 7
70	31 2	61 0

Such experiments, both without tissue and in the presence of boiled tissue (non-respiring) showed that the O_2 consumption was nearly twice that indicated by the analyses. This difference was probably not due to an accumulation of H_2O_2, small amounts of which are known to occur during the aerobic oxidation of ascorbic acid by copper salts, since the addition of liver catalase did not produce any appreciable evolution of gas when added from the side arm to the partially oxidized vitamin solutions. Irreversible oxidation of the vitamin is believed to be responsible for this difference. Subsequent to irreversible oxidation, by which the oxygen consumption is doubled, it is impossible to recover ascorbic acid by hydrogen sulfide reduction.

The technique of the experiments designed to study the direct effect of the vitamin on liver and kidney respiration was therefore (*a*) to estimate titrimetrically the loss of vitamin occurring *during* the respiration period, (*b*) to measure manometically the increased O_2 consumption due to the presence of vitamin in the medium, and (*c*) to compare the two measurements, assuming that the O_2 consumption equivalent to the vitamin loss calculated from the titration was twice the theoretical figure for reversible oxidation. Any excess in O_2 consumption found after including the above correction can be assumed to be a true effect of the vitamin on respiration. The respiration data with this correction are not recorded in detail, but a survey of 60 such experiments with two levels of added vitamin on normal and scorbutic liver and kidney may be summarized as follows: (*a*) With the lower level of vitamin in the medium, small (up to 15 per cent) and irregular increases in the corrected respiration occurred in some cases, while in others no effect could be found. There was no distinction between normal and scorbutic tissue in this respect. The results did not justify the conclusion that vitamin C addition *in vitro* produced an acceleration of respiration. (*b*) With the greater vitamin addition, the observed O_2 consumption was invariably balanced by the vitamin loss, with no noticeable difference between normal and scorbutic tissue.

Warburg's Indirect Method

The measurements of oxygen consumption and carbon dioxide production were carried out in Warburg square type vessels with a bicarbonate-Ringer's medium containing 0.2 per cent glucose in an atmosphere of 95 per cent O_2 and 5 per cent CO_2. Approximately 80 mg. of wet liver or 40 mg. of wet kidney were used in each of the vessel pairs. The tissues were placed in the medium during gas and temperature equilibration and allowed to respire for 2 hours. Details of the calculations are given in Dixon's "Manometric methods" (9), and the method of determining Q was the same as described previously. The results are recorded in Table III.

TABLE III

O_2 Consumption and CO_2 Production of Normal and Scorbutic Liver and Kidney (Measured in C.Mm. of Gas per Mg. of Dry Tissue per Hour)

Bicarbonate-Ringer's solution, pH 7.4, 95 per cent O_2 and 5 per cent CO_2 atmosphere.

	Normal			Scorbutic		
	Q_{O_2}	Q_{CO_2}	CO_2/O_2	Q_{O_2}	Q_{CO_2}	CO_2/O_2
Liver	4.0– 4.3(2)* 4.4– 5.2(10) 5.4(2)	2.9– 3.3(2) 3.4– 4.2(10) 4.3(2)	0.69–0.75(2) 0.76–0.84(10) 0.85–0.91(2)	5.3– 5.9(5) 6.0– 7.0(8) 7.5(1)	4.1– 4.4(3) 4.5– 5.5(7) 5.6– 6.3(4)	0.74–0.84(12) 0.87–0.88(2)
Average	4.8(14)	3.8(14)	0.80(14)	6.3(14)	5.1(14)	0.80(14)
Kidney	11.0–12.0(7) 12.1–13.3(4) 13.4–14.5(3)	10.8–11.5(3) 11.6–13.5(8) 13.6–15.2(3)	0.92–0.94(3) 0.95–1.00(11)	10.4–12.5(3) 12.6–14.5(7) 14.6–15.3(4)	11.6–13.6(11) 13.7–14.4(3)	0.89–0.94(10) 0.95–1.00(4)
Average	12.9(14)	12.5(14)	0.97(14)	13.5(14)	12.6(14)	0.93(14)

* The number of animals falling in each group is indicated in parentheses.

The oxygen consumption of scorbutic liver ($Q_{O_2} = 6.3$) was again significantly higher than that of normal liver ($Q_{O_2} = 4.8$). The carbon dioxide production increased proportionally, so that the ratio of CO_2/O_2 was the same in both normal and scorbutic liver. It may be noted that the oxygen consumption rate is somewhat higher when determined by this method than when measured by the Warburg direct method, but the indirect method is considered to represent physiological conditions more closely (9).

The slight rise in the oxygen consumption of scorbutic kidney with a resulting lower CO_2/O_2 ratio was not considered significant.

TABLE IV

Anaerobic Glycolysis of Normal and Scorbutic Liver and Kidney

Bicarbonate-Ringer's solution, pH 7.4, 95 per cent N_2 and 5 per cent CO_2 atmosphere.

$Q_G^{N_2}$, c.mm. CO_2 per mg. dry tissue per hr.

Liver		Kidney	
Normal	Scorbutic	Normal	Scorbutic
1 30-1 50(8)*	1 15(3)	5 65-6 10(2)	5 40(2)
1 60-1 70(4)	1 30-1 50(11)	6 10-6 85(8)	5 8-6 8(6)
			7 5-7 8(3)
Average 1 46(12)	1 37(14)	6 19(10)	6 45(11)

* The number of animals falling in each group is indicated in parentheses.

Apparently there was no extensive aerobic glycolysis, because the CO_2/O_2 ratio did not exceed unity in any case.

Anaerobic Glycolysis

The measurement of lactic acid production under anaerobic conditions was determined by the production of CO_2 from a bicarbonate-Ringer's solution at pH 7.4. Warburg square type vessels were used, filled with 95 per cent N_2 and 5 per cent CO_2 which had been freed from traces of O_2 by passage through a heated tube containing copper. The tissues were placed in the flasks during gas and temperature equilibration, and the gas mixture allowed to pass for 10 to 15 minutes, after which the flasks were shaken for 2 hours with readings at 15 minute intervals. Duplicate vessels were used throughout. The results of these experiments are recorded in Table IV.

Apparently ascorbic acid has no important part in the formation of lactic acid by the liver or kidney under anaerobic conditions.

Parallel experiments showed that the addition of vitamin C (0.1 or 0.4 mg. per ml.) *in vitro* had no effect upon anaerobic glycolysis. Under the conditions of the experiment, no loss of vitamin occurred in 2 hours.

TABLE V

Q_{O_2}, Q_{CO_2}, $Q_G^{O_2}$, and Respiratory Quotient of Normal and Scorbutic Liver, According to Dickens and Simer's Second Method

Bicarbonate-Ringer's solution, pH 7.4, 95 per cent O_2 and 5 per cent CO_2 atmosphere.

Normal				Scorbutic			
Q_{O_2}	Q_{CO_2}	R.Q.	$Q_G^{O_2}$	Q_{O_2}	Q_{CO_2}	R.Q.	$Q_G^{O_2}$
4.2	3.1	0.74	0.8	4.7	4.2	0.89	1.1
4.2	2.9	0.70	0.8	5.6	4.1	0.73	0.9
4.3	3.3	0.76	1.0	5.8	4.1	0.70	0.8
4.5	3.1	0.70	1.0	5.8	4.0	0.70	0.9
4.5	3.2	0.72	0.9	5.8	4.2	0.72	0.8
4.6	3.4	0.76	0.9	5.9	4.4	0.74	1.0
4.6	3.2	0.71	1.0	5.9	4.6	0.78	1.1
4.7	3.4	0.73	1.0	5.9	4.2	0.71	0.8
4.8	3.7	0.77	0.8	6.0	4.3	0.72	0.9
5.0	4.1	0.82	0.8	6.1	4.6	0.76	1.0
5.1	3.9	0.76	1.0	6.1	4.5	0.74	1.2
				6.2	4.8	0.77	1.0
				6.2	4.4	0.71	1.0
				6.4	4.6	0.72	0.9
				6.6	5.0	0.76	1.0
Average 4.6	3.4	0.74	0.90	5.9	4.4	0.75	0.96

Q_{O_2}, Q_{CO_2}, $Q_G^{O_2}$, and Respiratory Quotient of Normal and Scorbutic Liver by the Second Method of Dickens and Simer

Details of this method are given in the original article by Dickens and Simer (6) as well as in Dixon (9). The flasks contained 95 per cent O_2 and 5 per cent CO_2 (passed into the flasks for at least 10 minutes) and bicarbonate-Ringer's medium. Equal weights (approximately 120 mg.) of moist liver slices were placed in the appropriate vessels, after which the medium was added and the flasks were immediately transferred to the respirometer for gas and temperature equilibration. The oxygen consumption was allowed to proceed for 90 minutes, and 30 minutes was allowed for both liberation and absorption of CO_2 during the experiment. The results of these experiments are recorded in detail in Table V.

Again the O_2 consumption and CO_2 production of scorbutic liver gave values appreciably higher than those of normal liver. The aerobic glycolysis and respiratory quotient remained the same in both normal and scorbutic liver.

TABLE VI

Correlation of Scurvy Score with O_2 Consumption Rate of Liver

No. of animals	Scurvy score	Average Q_{O_2}	Per cent increase in Q_{O_2}*
41	0 (Normal)	4.5	
17	5-11	5.8	29
18	12-15	6.1	36
9	16-21	6 3	40

* Q_{CO_2} increases also (*cf.* Tables III and V) so that CO_2/O_2 and the true R.Q. remain unchanged.

It is of interest to compare these figures with the data obtained by using the other methods. The oxygen consumption rates of normal liver agree more closely with those obtained by Warburg's indirect method. The CO_2/O_2 ratios obtained by the Dickens and Simer method are lower than those determined by the Warburg indirect method because the latter include CO_2 arising from aerobic lactic acid production.

Q_{O_2} *and Severity of Scurvy*

The rates of oxygen consumption determined by Warburg's direct and indirect methods and the method of Dickens and Simer were combined and grouped according to the scurvy score of the animals (*cf.* Table VI). These averages, representing many animals, indicate a progressive increase in the oxygen consumption rate with the onset of scurvy. The same tendency was evident in each of the experimental methods, but individual variations from such a regular progression were fairly frequent.

It will be noted in Table VI that the change in Q_{O_2} is most rapid during the early stages of deficiency. This is of interest because it is known that the vitamin content of the tissues drops rapidly before the symptoms of scurvy are evident.

Borsook *et al.* (10) have suggested that the ascorbic acid-glutathione system might act catalytically between dehydrogenases and oxygen activators, and thus play an important rôle in tissue respiration. This study does not lend support to such a view, however.

SUMMARY

The oxygen consumption rate of liver increases noticeably (up to about 40 per cent) with the onset of scurvy, but there is no significant change in the rate for kidney cortex.

The carbon dioxide production of liver is also increased in scurvy, so that the CO_2/O_2 ratio as determined by the Warburg indirect method and the true R.Q. (Dickens and Simer) remain unchanged.

The anaerobic lactic acid formation in both liver and kidney, and the aerobic production of lactic acid by liver remain unchanged during the onset of scurvy.

The apparent increased oxygen consumption of liver and kidney due to the addition of vitamin C *in vitro* could not be attributed to an effect of the vitamin on tissue respiration. It was equivalent only to the oxidation of vitamin that occurred during the respiratory period. The effect was essentially the same with both normal and scorbutic liver and kidney.

BIBLIOGRAPHY

1. King, C. G., *Physiol. Rev.*, **16**, 238 (1936).
2. Harrison, D. C., *Biochem. J.*, **27**, 1501 (1933).
3. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **28**, 1014 (1934).
4. Phillips, P. H., Stare, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, **106**, 41 (1934).
5. Kato, Y., *Sei-i-kai Med. J.*, **55**, 2037 (1936); *Chem. Abst.*, **31**, 1860 (1937).
6. Dickens, F., and Simer, F., *Biochem. J.*, **25**, 973 (1931).

7. Sherman, H. C., and Smith, S. L., The vitamins, American Chemical Society monograph series, New York, 2nd edition (1931).
8. Musulin, R. R., and King, C. G., *J. Biol. Chem.*, **116**, 409 (1936).
9. Dixon, M., Manometric methods, New York (1934).
10. Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, **117**, 237 (1937).

THE ERGOT ALKALOIDS

XII. THE SYNTHESIS OF SUBSTANCES RELATED TO LYSERGIC ACID*

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In a series of papers during the past few years Jacobs and Craig have shown that the ergot alkaloids are derivatives of a unique acid base, lysergic acid, in which the latter or an isomer is conjugated with certain amino acids or substances which can be derived from them, such as 2-aminopropanol-1, pyruvic acid, and isobutyrylformic acid. Since lysergic acid is thus the common characteristic constituent of these alkaloids, the determination of its structure became at once a major issue in the ergot alkaloid problem. Degradation studies combined with a study of its characteristic groupings and other properties made it possible to derive a very probable structure for lysergic acid, as given in Formula I.[1] This formula appears to explain satisfactorily all of the observations which have been made. An uncertainty, however, remains as to the exact position to which the carboxyl group can be assigned. All evidence in this case points to certain positions on Ring D, preferably (7) or (8). It has therefore been of importance and interest to obtain a direct check on the validity of this formulation by synthesis.

There appears to be no recorded experience in regard to the possibility of the formation of the tricyclic system represented by Rings A, B, and C, not to speak of the tetracyclic structure A, B, C, D given in Formula I. As a first step, the synthesis of *3,4-trimethylene indole* (Formula II) was attempted. This was accomplished by the reduction of naphthostyril with sodium and butyl alcohol. An expected by-product of the reaction was also encountered and was separated from the indole derivative by virtue of its basic properties, namely 1-hydroxymethyl-8-amino-1,2,3,4-tetrahydronaphthalene (Formula III). The identities of these substances were established by their production by a different procedure; namely, reduction of the methyl ester of 8-amino-1,2,3,4-tetrahydro-1-naphthoic acid, by the aromatic character of the amino group of the second (basic) reduction product, and by the characteristic indole reactions given by trimethylene indole. The latter, however, did not give the deep blue Keller test so characteristic of lysergic acid and its derivatives; instead a deep red-brown color was produced.

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* Jacobs, W. A., and Gould, R. G., Jr., *Science*, **85**, 248 (1937).

[1] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **115**, 227 (1936).

As a next step the attempt was made to realize the assumed 4-ring system of lysergic acid. This was actually accomplished as follows: The first objective was *5,6-benzoquinoline-7-carbonic acid* (Formula IV), which was obtained by a series of reactions beginning with 1,8-naphthalic acid. This acid was converted into 3-nitro-1-naphthoic acid according to Leuck, Perkins, and Whitmore,[2] and yielded 3-amino-1-naphthoic acid on reduction. The amino acid, when subjected to the Skraup synthesis, gave the desired benzoquinoline carbonic acid which was further characterized by its salts and *ethyl ester*. On nitration, benzoquinoline carbonic acid fortunately appeared to give mainly *3′-nitro-5,6-benzoquinoline-7-carbonic acid*. *3′-Amino-5,6-benzoquinoline-7-carbonic acid* resulted on reduction of the nitro acid. The amino acid readily formed the *benzoquinoline lactam* (Formula V).

As in the case of naphthostyril, the lactam base on reduction with sodium in butyl alcohol gave a mixture of the desired tetracyclic indole derivative (Formula VI) and the by-product due to reductive cleavage of the lactam grouping to the aromatic amino alcohol, *viz.* *3′-amino-7-hydroxymethyl-1,2,3,4,7,8,9,10-octahydro-5,6-benzoquinoline* (Formula VII). These substances were separated by virtue of their differential solubilities in ether.

For the above tetracyclic indole we propose the trivial name *ergoline* and have followed in Formula VI the same numbering used in the case of the formula for lysergic acid.[1] In the case both of the latter and of the amino alcohol simultaneously produced, it

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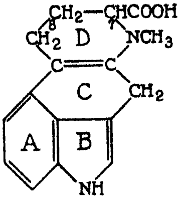
[2] Leuck, G. L., Perkins, R. P., and Whitmore, F. C., *J. Am. Chem. Soc.*, **51**, 1834 (1929).



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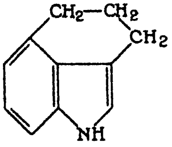

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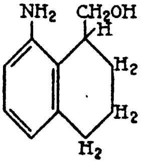

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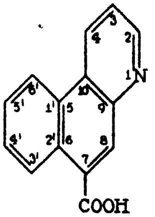

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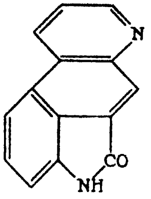

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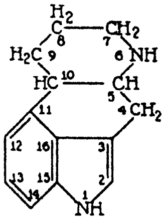

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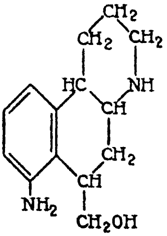

VII

is obvious that each contains several asymmetric centers. Therefore, although not certain, it is possible that the substances isolated by us may still be non-homogeneous in the sense of being mixtures of epimers.

Of greatest interest, however, is not only the synthetic realization of the assumed tetracyclic system of lysergic acid (dihydrolysergic acid) but the color reactions produced by ergoline. The reactions with dimethylaminobenzaldehyde (as an indole) are identical with those given by lysergic acid. In the case of the Keller test there is a very close approach to that which is so characteristic of lysergic acid. In intensity and speed of development it is indistinguishable from the latter. However, instead of a deep pure blue, the color is more of a violet-blue. This slight difference could doubtlessly be caused either by the absence of the carboxyl group or of the N-methyl group of lysergic acid or of both.

Finally, catalytic hydrogenation of the above lactam base has resulted in only partial reduction to a tetrahydroquinoline derivative, *viz. 3'-amino-1,2,3,4-tetrahydro-5,6-benzoquinoline-7-carbonic acid lactam.* On reduction of this tetrahydro lactam with sodium and butyl alcohol, again a mixture of ergoline and the corresponding amino alcohol was obtained.

There is now in progress in this laboratory a logical extension of this synthetic work both in regard to methods of procedure and to the inclusion of substitution products of ergoline and finally, it is hoped, of the synthesis of lysergic acid or its derivatives.

EXPERIMENTAL

Reduction of Naphthostyril—10 gm. of sodium were added to a boiling solution of 2 gm. of naphthostyril in 200 cc. of dry butyl alcohol and the mixture was shaken vigorously. After the sodium had dissolved, water was added and the butyl alcohol was removed in a vacuum. The residual aqueous solution was extracted with ether, and the extract in turn was shaken with 2 per cent hydrochloric acid.

3,4-Trimethylene Indole—The above ether solution gave 0.8 gm. of the indole as a dark red oil, which crystallized when allowed to cool and scratched. It was purified by sublimation at 120–140° at 0.3 mm. pressure. The colorless, crystalline sublimate on recrystallization from low boiling petroleum ether separated as long, colorless prisms which melted at 58.5–59°.

$C_{11}H_{11}N$. Calculated. C 84.08, H 7.07, N 8.92
Found. " 84.18, " 7.18, " 8.97

Trimethylene indole has an odor resembling more that of α-naphthylamine than that of indole or skatole. It is very soluble in ether, alcohol, and benzene; moderately so in warm petroleum ether and ligroin; and practically insoluble in water and dilute acids. It gives an intense reddish violet color with *p*-dimethylaminobenzaldehye and hydrochloric acid. A reddish brown color is produced with Keller's reagent, which is quite different from the deep blue color given by lysergic acid and the ergot alkaloids.

Trimethylene indole picrate was obtained from alcohol as long, dull red needles which melted at 164–166°.

$C_{11}H_{11}N \cdot C_6H_3O_7N_3$. Calculated. C 52.8, H 3.65, N 14.5
Found. " 52.93, " 3.46, " 14.43

1-Hydroxymethyl-8-Amino-1,2,3,4-Tetrahydronaphthalene—This by-product in the formation of trimethylene indole was contained in the 2 per cent hydrochloric acid solution described above. After the dissolved ether was removed and the solution made alkaline, the product crystallized. The yield was 0.8 gm. of crude substance from 2 gm. of naphthostyril. Crystallized from alcohol and then from ether, it formed square, colorless platelets which melted at 111–112°.

$C_{11}H_{16}ON$. Calculated, C 74.6, H 8.53; found, C 74.38, H 8.58

The fact that the amino alcohol after diazotization coupled to form dyes showed that the ring containing the amino group is aromatic. Attempts to transform the amino alcohol into trimethylene indole were unsuccessful.

The hydrochloride was obtained from a small volume of dilute hydrochloric acid.

$C_{11}H_{16}ON \cdot HCl$. Calculated, C 61.9, H 7.54; found, C 62.12, H 7.56

The picrate formed light yellow prisms from alcohol, which melted at 206–207°.

The *N-benzoyl derivative* was prepared by treatment of the amino alcohol in benzene solution with excess benzoyl chloride and potassium carbonate. After recrystallization from alcohol it melted at 195.5–197°.

$C_{18}H_{19}O_2N$. Calculated. C 76.9, H 6.84, N 4.98
Found. " 76.50, " 6.78, " 5.25

The properties of the substance made it apparent that acylation occurred on the amino group.

Both trimethylene indole and hydroxymethyl amino tetrahydronaphthalene were obtained also from the methyl ester of 8-amino-1,2,3,4-tetrahydro-1-naphthoic acid. This substance was prepared according to Schroeter and Rössler[3] who reported a melting point of 53–54°. Our preparation, however, after repeated recrystallization from petroleum ether melted at 75–76°.

$C_{12}H_{15}O_2N$. Calculated, C 70.30, H 7.36; found, C 70.30, H 7.47

The ester was reduced with sodium and butyl alcohol, as described above for naphthostyril, and gave the same reduction products. 0.2 gm. of the ester gave 70 mg. of trimethylene indole and 100 mg. of the amino alcohol.

5,6-Benzoquinoline-7-Carbonic Acid—3-Nitro-1-naphthoic acid was prepared from 1,8-naphthalic acid according to Leuck, Perkins, and Whitmore.[2] After recrystallization from glacial acetic acid it was then reduced to the amino acid with ferrous sulfate and ammonia. Since the free amino acid is unstable, it was found advantageous to isolate it as the sparingly soluble sulfate.

22 gm. of nitronaphthoic acid were dissolved in 300 cc. of 15 per cent ammonia, and 180 gm. of ferrous sulfate in 300 cc. of water were added. The mixture was brought to a boil and then filtered. The clear, light yellow filtrate on acidification with sulfuric acid yielded the sulfate which was collected in almost theoretical yield.

A mixture of 19 gm. of the sulfate, 13 gm. of sulfuric acid, 25 gm. of dry glycerol, and 5 gm. of nitrobenzene was heated for 5 hours at 170–180°. The reaction mixture after dilution with 200 to 300 cc. of water was boiled and filtered. The acid filtrate was extracted with ether, then made alkaline, filtered, and again extracted with ether. Finally, the aqueous solution was made just acid to litmus with acetic acid, and quickly filtered, or decanted, from precipitated tar. The benzoquinoline carbonic acid then crystallized when scratched and allowed to stand. Occasionally, however, the substance crystallized immediately with the tar. In such a case, repeated recrystallization as the hydrochloride from dilute hydrochloric acid proved necessary.

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[3] Schroeter, G., and Rössler, H., *Ber. chem. Ges.*, **35**, 4222 (1902).

In either case, final purification was accomplished by treatment of the dilute hydrochloric acid solution with norit, followed by cautious addition of sodium acetate solution. The colorless, microcrystalline powder so obtained melted with decomposition at 298–300°, and with the appearance of the odor of benzoquinoline.

$C_{14}H_9O_2N$.	Calculated.	C 75.4, H 4.04, N 6.3
	Found.	" 75.38, " 3.85, " 6.44
	"	" 75.17, " 4.22

Benzoquinoline carbonic acid is soluble in dilute mineral acid and alkali, and in pyridine; it is sparingly soluble in acetic acid and alcohol. It is a stronger acid than base and is almost completely precipitated at a pH just acid to Congo red.

The hydrochloride crystallized from dilute hydrochloric acid in microscopic needles.

$C_{14}H_9O_2N \cdot HCl$. Calculated, C 65.00, H 3.91; found, C 65.10, H 3.81

The ethyl ester was prepared by refluxing a suspension of the acid in absolute alcohol saturated with HCl for several hours and until solution was complete. Although on concentration the ester hydrochloride separated in transparent rhombs, it was not isolated as such but converted into the free ester. The latter, after recrystallization from alcohol, melted at 104–105°.

$C_{16}H_{13}O_2N$. Calculated, C 76.50, H 5.21; found, C 76.40, H 5.25

3′-Nitro-5,6-Benzoquinoline-7-Carbonic Acid—4 gm. of finely powdered benzoquinoline carbonic acid were gradually added to 16 cc. of fuming nitric acid (sp. gr. 1.58) cooled in ice, and the mixture was then allowed to stand at room temperature overnight.

Concentration of the solution *in vacuo* at room temperature gave large, colorless crystals of the nitrate of the nitro compound. The salt, however, hydrolyzed on washing with water. The nitro compound was then dissolved in dilute sodium hydroxide solution and reprecipitated with acetic acid, as a colorless microcrystalline powder melting at 310°.

$C_{14}H_8O_4N_2$. Calculated. C 62.7, H 3.01, N 10.45
Found. " 62.84, " 3.37, " 10.20

Nitrobenzoquinoline carbonic acid gives silvery leaflets of the sodium salt from dilute sodium hydroxide solution. The hydrochloride crystallizes from 15 per cent hydrochloric acid as long needles.

For the next step, however, it was found advantageous to pour the nitration mixture directly onto ice, and to collect the precipitate with water. The material thus obtained was light tan in color and practically pure. The yield was almost theoretical.

3'-Amino-5,6-Benzoquinoline-7-Carbonic Acid Lactam—The above nitro derivative was reduced with ferrous sulfate and alkali to the amino acid which was then lactamized by treatment with hydrochloric acid in the following steps.

2.7 gm. of nitro compound were dissolved in 130 cc. of 5 per cent sodium hydroxide and warmed. A hot solution of 20 gm. of ferrous sulfate in 50 cc. of water was then added, and the mixture was brought to a boil and then filtered. The iron precipitate was reextracted with hot dilute sodium hydroxide solution. The combined clear, yellow filtrates were made strongly acid with hydrochloric acid. On standing, the hydrochloride of the lactam crystallized as red needles. The salt was recrystallized from dilute hydrochloric acid.

$C_{14}H_8ON_2 \cdot HCl$. Calculated, C 65.50, H 3.54; found, C 65.51, H 3.58

The lactam base obtained by decomposition of the salt was purified by recrystallization from alcohol with bone-black. It separated as long, silky, bright yellow needles which melted at 280°.

$C_{14}H_8ON_2$. Calculated, C 76.40, H 3.66; found, C 76.47, H 3.53

The over-all yield from 5,6-benzoquinoline-7-carbonic acid was about 65 per cent, which indicates that the nitration must give mainly the 3'-nitro derivative.

The lactam ring is readily opened by heating with strong alkali and is again closed on acidification with mineral acids even at room temperature.

The free amino acid was obtained, however, from the sodium salt with dilute acetic acid in the cold. The dry acid which is carmine-red in color changes to the yellow lactam on heating. It can be diazotized and coupled with α-naphthol, whereas the lactam gives a negative diazo reaction.

Reduction of 3′-Amino-5,6-Benzoquinoline-7-Carbonic Acid Lactam—2 gm. of sodium were added to a boiling solution of 0.45 gm. of the lactam in 40 cc. of dry butyl alcohol and the mixture was shaken vigorously. The latter changed instantly to a brilliant scarlet and then finally became colorless. After addition of water the butyl alcohol was removed *in vacuo*. The yellowish or brownish yellow oil was dissolved in chloroform, washed with water, and the solvent removed *in vacuo*. About 0.4 gm. of a partly crystalline residue was obtained, from which the following two substances were isolated.

Ergoline—The above residue was extracted with two 10 cc. portions of warm ether. After standing at 0°, the ether solution was filtered and concentrated to small volume. On chilling, crude ergoline crystallized. It was recrystallized from 60 per cent methyl alcohol and then from ether. The base was obtained as a white crystalline powder, melting over a rather wide range, *i.e.* 175–183°, and possibly consisted of stereoisomers.

It gave an intense indole color test with *p*-dimethylaminobenzaldehyde and hydrochloric acid. With this reagent and concentrated sulfuric acid (the van Urk test), it gave colors indistinguishable from those given by lysergic acid. With Keller's reagent a test very similar to, but not identical with that given by lysergic acid was obtained, in which the color was a deep blue-violet instead of the usual deep blue.

Ergoline is unstable to air and to acids. It is very soluble in alcohol, chloroform, and benzene; but moderately soluble in ether; and insoluble in water and petroleum ether.

$C_{14}H_{16}N_2$.	Calculated.	C 79.2,	H 7.58,	N 13.2
	Found.	" 78.96,	" 7.20,	" 13.12
	"	" 78.84,	" 7.44	

The hydrochloride was obtained from the crude as well as the pure base by crystallization from 10 per cent hydrochloric acid.

When recrystallized from dilute methyl alcohol, it formed a colorless microcrystalline powder which is practically insoluble in water.

$C_{14}H_{16}N_2 \cdot HCl$. Calculated. C 67.60, H 6.88
Found. " 67.60, " 6.56
" " 67.43, " 6.68

3'-Amino-7-Hydroxymethyl-1,2,3,4,7,8,9,10-Octahydro-5,6-Benzoquinoline—This by-product of the preparation of ergoline was obtained from the fractions less soluble in ether. This residue described above was dissolved in a large volume (300 to 400 cc.) of ether. On concentration to half volume and cooling, the amino alcohol separated in fairly pure form. It gave negative indole color tests, and after diazotization coupled with α-naphthol. It melted at 80-85° and possibly consisted of stereoisomers.

$C_{14}H_{20}ON_2$. Calculated, C 72.5, H 8.7; found, C 71.88, H 8.5

The dihydrochloride separated from a small volume of dilute hydrochloric acid as silvery leaflets. It was recrystallized from acetone containing a little dilute hydrochloric acid.

$C_{14}H_{20}ON_2 \cdot 2HCl \cdot H_2O$. Calculated. C 52.00, H 7.50
Found. " 51.39, " 7.64

3'-Amino-1,2,3,4-Tetrahydro-5,6-Benzoquinoline-7-Carbonic Acid Lactam—220 mg. of the above amino lactam were dissolved in hot dilute HCl and kept warm to prevent crystallization of the hydrochloride. The solution was hydrogenated with 50 mg. of Adams and Shriner's catalyst. 2 moles of H_2 were absorbed in about an hour, during which the color of the solution changed from red to yellow. Cautious neutralization with sodium acetate solution caused crystallization of the tetrahydro compound as pale yellow needles. After crystallization from alcohol, it melted at 248-249°.

$C_{14}H_{12}ON_2$. Calculated, C 75.00, H 5.40; found, C 74.61, H 5.23

The hydrochloride crystallized as yellow needles from dilute hydrochloric acid.

THE FORMATION OF DOPA BY THE EXPOSURE OF TYROSINE SOLUTIONS TO ULTRAVIOLET RADIATION

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Protein solutions absorb oxygen and undergo an increase in the absorption of ultraviolet light when they are exposed to ultraviolet radiation (3, 5, 6, 8, 9). It is also known that egg albumin utilizes oxygen and, under certain conditions of pH, has an increased absorption in the ultraviolet region of the spectrum during exposure to α particles (1). These facts led the author to postulate that tyrosine could be converted to dopa (*l*-3,4-dihydroxyphenylalanine) by radiant energy (1).

A colorimetric procedure for the determination of dopa in the presence of tyrosine has been described by the author (2). The color produced by dopa, when analyzed spectrophotometrically, permits the identification of this compound. The results obtained by comparing the color produced by dopa and by a tyrosine solution which had been exposed to ultraviolet radiation are illustrated in Fig. 1. This curve indicates that some of the tyrosine has been converted to dopa by the radiant energy.

Fig. 2 indicates that not all the tyrosine destroyed can be recovered as dopa. Experiments by the author have indicated, however, that dopa is also destroyed by ultraviolet radiation. The shape of the tyrosine destruction curve in Fig. 2 suggests that the reaction is monomolecular. This type of reaction is also indicated by experiments in which solutions of differing tyrosine concentrations were exposed to ultraviolet radiation for the same time interval. In each case, the percentage of tyrosine destroyed was the same.

The destruction of tyrosine did not occur in the absence of air.

Some destruction occurred through glass filters, but the destruction was much more marked when quartz filters were used.

All the solutions irradiated assumed a reddish brown color. No aldehyde was formed, since negative tests were obtained with Schiff's reagent. All irradiated solutions gave faintly positive tests with Nessler's reagent.

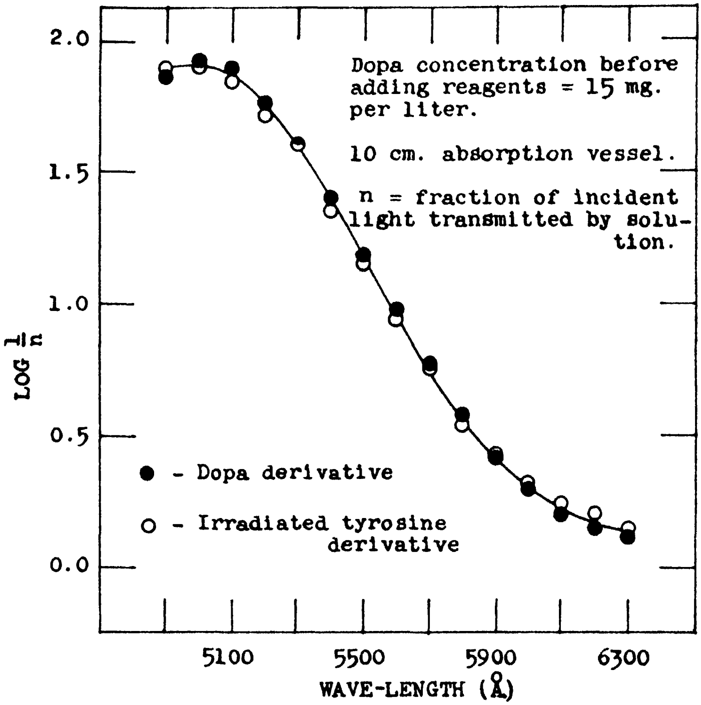

FIG. 1. Absorption spectra of irradiated tyrosine and dopa derivatives

The work of Bloch (4), Raper (7), and their coworkers has shown that tyrosine is the probable precursor of melanin, and that dopa is the first product produced from tyrosine in the process of melanin formation. Raper states, however, that the mechanism which converts tyrosine to dopa in the higher animals is unknown. Tyrosinase has been found so far only in plants, bacteria, and the lower forms of animal life. The results given in this paper suggest that one of the mechanisms whereby radiant energy causes increased melanin formation in the skin is the production of dopa from tyrosine, the dopa then being converted to melanin by the dopa oxidase of the melanoblasts.

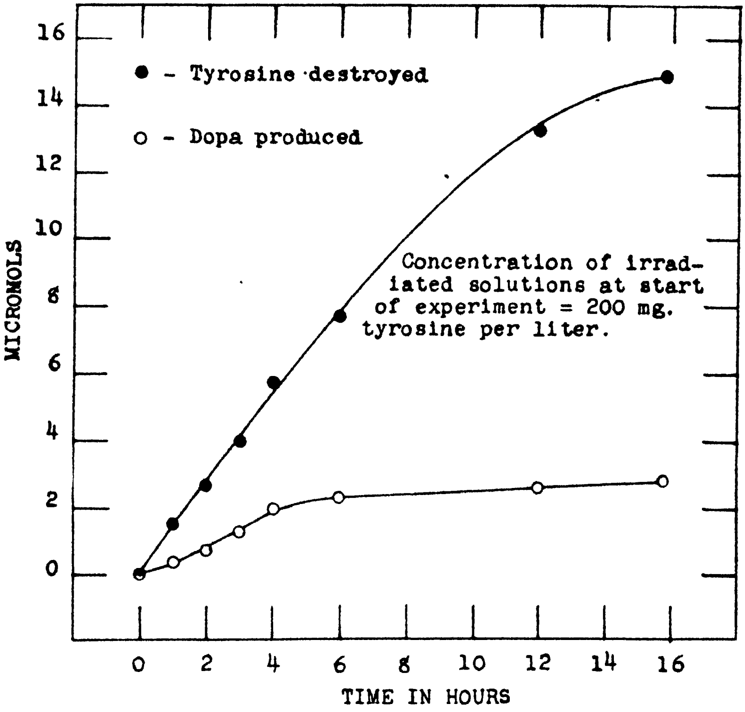

Fig. 2. Production of dopa and destruction of tyrosine as functions of time.

BIBLIOGRAPHY

1. Arnow, L. E., *J. Biol. Chem.*, **110**, 43 (1935).
2. Arnow, L. E., *J. Biol. Chem.*, **118**, 531 (1937).
3. Becker, J. P., and Szendrö, P., *Arch. ges. Physiol.*, **228**, 755 (1931).
4. Bloch, B., *Z. physiol. Chem.*, **98**, 226 (1917).
5. Harris, D. H., *Biochem. J.*, **20**, 288 (1926).
6. Hausmann, W., and Spiegel-Adolf, M., *Klin. Woch.*, **6**, 2182 (1927).
7. Raper, H. S., *Physiol. Rev.*, **8**, 245 (1928).
8. Spiegel-Adolf, M., and Krumpel, O., *Biochem. Z.*, **190**, 28 (1927); **208**, 45 (1929).
9. Spiegel-Adolf, M., and Oshima, Z., *Biochem. Z.*, **208**, 32 (1929).

DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

IX. THE CONVERSION OF STEARIC ACID INTO PALMITIC ACID IN THE ORGANISM*

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The saturated fatty acids of mammals consist of a mixture of the two acids, stearic acid, $C_{18}H_{36}O_2$, and palmitic acid, $C_{16}H_{32}O_2$, together with small amounts of other acids of lower or higher molecular weight. Channon *et al.* (1) have demonstrated that rats fed on various diets show a tendency to keep the ratio of palmitic acid to stearic acid constant.

On a fat-free diet mammals synthesize almost all their fatty acids from carbohydrates (or amino acids) and, as the animals always contain a mixture of all acids characteristic for the species, they must have their ultimate origin, under these conditions, in carbohydrates or amino acids. The question arises whether all these acids are directly synthesized or may also be formed by the conversion of one acid into another. The occurrence of saturation (2) and desaturation (3) has already been demonstrated in previous papers. The aim of this study is to investigate whether the animal is able to shorten the chain of stearic acid by 2 carbon atoms in order to prepare palmitic acid. The well known experiments on β oxidation suggest that such a reaction may take place. However, no methods have been available for investigating this special process.

In this paper we describe the biological degradation of stearic acid to palmitic acid. From mice which were fed deuterostearic acid there was isolated deuteropalmitic acid. The palmitic acid

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was obtained by fractional distillation of the methyl esters of the saturated acids from the mice. We consider this result a proof of the ability of the organism to prepare palmitic acid from stearic acid.

We have reported that after feeding fatty acids, whether saturated or unsaturated, a large quantity of the administered acid was deposited in the fat tissue. The same was found in the present experiment: the stearic acid fraction from the distillation contained a considerable amount of deuterium, obviously owing to the deposition in the animal of the unchanged stearic acid. As was to be expected, the deuterium content of the palmitic acid fraction was much lower than that of the stearic acid fraction.

The fractional distillation of the methyl esters of the higher fatty acids is carried out at high temperatures (150° and higher). It had to be demonstrated that even at such high temperatures deuterium bound to carbon in fatty acids is stable and does not exchange with the hydrogen of another fatty acid. Two experiments were carried out to exclude the possibility of such an exchange. A mixture of methyl deuterostearate and ordinary deuterium-free methyl palmitate was fractionated in our distillation apparatus. The methyl palmitate so obtained contained no trace of deuterium. In a second experiment, deuterostearic ester was mixed with the esters of saturated acids obtained from mice which had not received any deutero compounds. The first seven fractions, all of which consisted of palmitic acid, were free of deuterium. Both experiments definitely exclude any exchange between the deuterium of the stearic acid and the hydrogen of palmitic acid at the temperature of the distillation (150-200°). The deuterium content of the palmitic acid from the animals which were fed deuterostearic acid could not, therefore, have had its origin in an exchange with the deuterostearic acid.

In all work on the biological conversion of a deutero Substance A into a deutero Substance B (in the present case, deuterostearic into deuteropalmitic acid), the latter must always be rigorously purified so as to remove all traces of Substance A. The sensitivity of the analytical method is such that even slight contamination with Substance A might lead to erroneous interpretations. When Substances A and B have similar physical and chemical properties (as in the case of the fatty acids), this may involve great difficulties or actually be impossible. The problem is further complicated by the fact that one cannot rely upon the customary criteria of purity (melting point, molecular weight, elementary analysis, etc.) to exclude the presence of small amounts of contaminating substances. The presence of less than 5 per cent stearic acid in palmitic acid can hardly be detected by the usual laboratory procedures.

In such cases, in which Substance B has to be freed from traces of a deutero Substance A, a new general principle can be employed. It is based upon the fact that the deutero compound has the same properties as the normal one, and the two are therefore inseparable by any of the ordinary laboratory procedures. After Substance B is separated from Substance A as well as possible by any of the routine methods (precipitation, crystallization, fractional distillation, etc.), a large excess of the normal non-deuterium-containing analogue of Substance A is added to B. By this the contaminating deutero Substance A is diluted by the normal analogue added. Substance B is now again separated from A. The deuterium content of the contaminating Substance A has now been diluted proportionally to the amount of normal A added. By repeating this procedure it is possible to reduce it to practically zero. While this procedure does not remove all traces of Substance A from B, one substitutes normal Substance A for deutero Substance A.

This principle of "washing out" deutero substances was first used in our studies of desaturation (3), in which it was impossible to make a clear cut separation of the unsaturated acids from the saturated ones. The deutero-saturated acids were "washed out" by repeated addition of the normal saturated acids and precipitation as lead salts. In the present work the same principle was used for the removal of deuterostearic acid from the palmitic acid. After the first fractional distillation a large excess of normal stearate was added to the palmitate and the distillation repeated. The deuterium content of the stearate fraction was diluted about 50-fold, to a negligible value.

EXPERIMENTAL

The best method for the separation of higher fatty acids is the fractional distillation of their methyl esters under reduced pressure. A distillation apparatus was built with which palmitic acid could be isolated from stearic acid or from mixtures of other saturated acids. The amount required for fractionation was 1 to 3 gm.

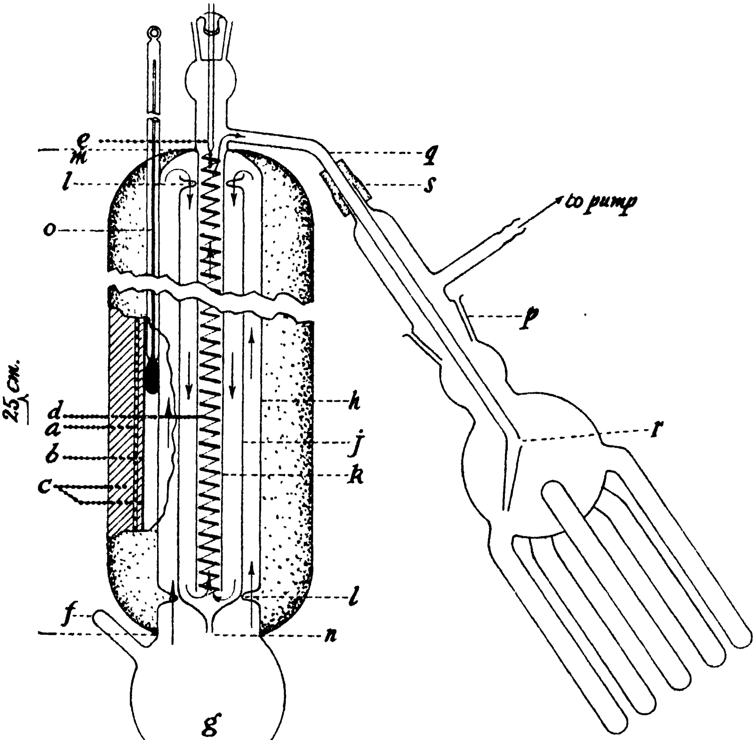

Fig. 1. Distillation apparatus for the fractionation of fatty acid esters. An explanation of the letters is given in the text.

Description of Distillation Apparatus—A diagram of the still is shown in Fig. 1. The still consists of three concentric tubes *h*, *j*, and *k*. The inner tube *k* (6 mm. inside diameter) is sealed to *h* (18 mm. inside diameter) at the ring seal *m*. The middle tube *j* (12 mm. inside diameter) is supported by the identations *l*. The middle tube *j* is drawn down to a tip *n* of about 1 mm. in diameter. The bottom of tube *k* is about 1 cm. above the beginning of the constriction. If this distance is too small, the column will flood. A close fitting wire helix *d* is placed in the inner tube *k*. The condensed liquid flows down the entire length of the helix. This allows good contact between the liquid and the vapor. The helix is made by winding No. 24 nichrome wire on a rod. It is then stretched until there are about seven turns to the inch. It should fit snugly into *k*. The thermocouple *e*, which measures the vapor temperature, is placed just at the top of the helix *d*. To facilitate cleaning the still, the thermocouple is attached by means of a No. 7 interchangeable ground joint. It is sealed into the joint with picein wax. We have used a copper-constantan couple, which was made according to the directions of Foote, Fairchild, and Harrison (4). The column is jacketed with two layers of asbestos *c* and heated by the nichrome wire *a* to avoid heat loss. A thermometer *o* is placed at the middle of the column and the entire column is wrapped with copper-foil *b*. It is covered with a thin layer of asbestos paper and a No. 28 nichrome heating coil is put on (four turns to the inch). The coil must start just below the side arm and extend down to the bottom indentations *l*. A thick layer of asbestos paper is then put on. The asbestos covering is finally painted with sodium silicate. The receiver is a 50 cc. round bottom flask to which are sealed six tubes (10 cm. × 6 mm.). The flask can be rotated on the greased joint *p*, so that different fractions can be taken without interrupting the distillation. The side arm *q* (5 mm. inside diameter) has a small hole *r*, through which the column can be evacuated without spraying the drop at the tip of the side arm. The entire receiving part is connected with the side arm by the short section of rubber tubing *s*.

The relation between the temperature of the column as given by the thermometer and the current in the coil *a* is first determined.

For the fractionation the ester mixture is placed in the flask *g* (10 cc.) through the side arm *f*, which is then sealed off. The system is evacuated by an oil pump to about 0.05 mm. pressure. A lower pressure is not advisable, as the efficiency of the column drops off. The current in the heating coil is set to bring the column up to about 110°. The flask *g* is immersed in a Wood's metal bath and the temperature slowly raised until the vapors enter the column and raise its temperature, as shown at the thermometer *o*. The current is now adjusted so that it alone would hold the temperature 5–10° below the temperature read at the thermometer. This adjustment must be made during the whole course of the distillation as the temperature of the column rises. The function of the heating coil is to reduce the heat loss of the column to a minimum but not to superheat the vapors. The vapors follow the path indicated by the arrows. The condensed liquid flows down the helix *d* and drops back into the flask *g* through the tip *n*. The rate of heating is adjusted so that 30 to 50 drops return to the flask *g* for each drop collected at the receiver. Reducing the reflux ratio (too rapid distillation) destroys the distillation efficiency. Not more than 150 to 200 mg. should be collected per hour. The hold-up of the still is about 300 mg. It can be reduced by superheating the column by the coil *a* at the end of the fractionation, but the fractionation efficiency is lowered thereby.

Table I

Fractionation of a Mixture of Methyl Palmitate and Methyl Stearate

Normal methyl palmitate, 1.074 gm.; methyl deuterostearate (3.38 atom per cent deuterium), 0.964 gm.

Fraction No.	Weight	Melting point	Deuterium content
	mg.	*°C.*	*atom per cent*
1	200	29.4–29.8	0.00
2	442	29.6–29.8	0.07
3	375	27.4–27.8	1.14
Residue			3.0

The physical constants of the fractions are obtained after the tubes containing the samples are cut off from the bulb.

Fractionation of Artificial Mixtures of Fatty Acid Esters

Preliminary experiments were carried out to determine the efficiency of the still and to investigate whether "stably bound" deuterium of one fatty acid would exchange with the hydrogen of another fatty acid at the high temperature necessary for distillation.

Fractionation of Methyl Palmitate and Methyl Deuterostearate—A mixture of 1.074 gm. of methyl palmitate and 0.964 gm. of methyl deuterostearate, containing 3.38 atom per cent deuterium, was fractionated. In this first crude experiment four large fractions were secured and the fractions characterized by their melting points. Methyl palmitate has the melting point 29.5° and methyl stearate, 38.0°. The melting points and the deuterium content are given in Table I. Fraction 1, consisting of pure methyl palmitate, did not contain any trace of deuterium. The deuterium of the stearate could therefore not have exchanged with the hydrogen of the palmitate. The small deuterium content of Fraction 2 is due to a small amount of stearate carried over (approximately 2 per cent). The melting point is not affected by this contamination.

TABLE II

Fractionation of Mixture of Methyl Esters of Saturated Fatty Acids

Esters from mice acids, 2.506 gm.; methyl deuterostearate (7.13 atom per cent deuterium), 0.516 gm.

Fraction No.	Weight	Melting point	Deuterium content
	mg.	*°C.*	*atom per cent*
1	208	15.8-21.0	0.00
2	193	27.4-28.8	0.00
3	370	29.4-29.8	
4	28	29.2-29.8	
5	303	29.1-29.4	
6	259	29.0-29.3	0.00
7	247	29.0-29.5	
8	174	25.0-25.4	0.35
9	158	32.3-32.5	2.05
10	183	30.9-31.2	2.66
Residue	680	32.0-33.0	4.13

Fractionation of Methyl Esters of Saturated Acids of Mouse Fat Mixed with Deuterostearic Acid—2.506 gm. of methyl esters of the saturated acids obtained from normal mice were mixed with 0.516 gm. of methyl deuterostearate. The mixture was separated into ten fractions and a residue of 680 mg. The data are given in Table II. The low melting points of Fractions 1 and 2 are due to the presence of acids of lower molecular weight. Fractions 3 to 7 have the correct melting point of palmitic acid. For deuterium analysis Fractions 2, 3, 4, and 5 and Fractions 6 and 7 were pooled. They, as well as Fraction 1, did not contain any deuterium. The presence of 1 per cent stearic acid would have been detected, since the deuterium content of the stearic acid in this mixture must have been greater than 4.13 atom per cent (deuterium content of residue). Fractions 8, 9, and 10 and the residue have increasing amounts of deuterium owing to the presence of stearic acid. This is in agreement with the changes of the melting points.

The recovery of fatty acid esters and of deuterium after fractionation was satisfactory. From 3.022 gm. of esters there were recovered 2.803 gm. (93 per cent). The apparent loss was incurred by the removal of small samples for melting point determinations before weighing the fractions. The starting material contained 0.516 gm. of methyl stearate with a deuterium content of 7.13 atom per cent. The amount of deuterium in the different fractions and residue is equivalent to 0.530 gm. (103 per cent) of methyl stearate of the same deuterium content. The errors of all the deuterium determinations enter into this value.

TABLE III

Fractionation of Fatty Acids from Mice Given Deuterostearic Acid

Fraction No.	Weight	Melting point	Deuterium content
	mg.	*°C.*	*atom per cent*
1	231	19.0-19.6	0.07
2	404	28.0-28.8	0.24
3	389	29.8-30.0	
4	349	29.5-29.8	0.43
5	495	29.0-29.3	
6	161	25.8-26.0	0.62
7	271	35.8-36.0	2.43
8	85		2.8
Residue	1400	34.4-35.0	2.53

Feeding of Mice with Ethyl Deuterostearate

Ten male mice were given a diet of whole wheat bread mixed with 8 per cent ethyl deuterostearate, which was obtained by hydrogenating with deuterium a mixture of linoleic and oleic acids. The ethyl stearate contained 7.00 atom per cent deuterium. The ester was prepared from the same stearic acid as used for the fractionation of the artificial mixtures. At the end of 5 days the mice were killed and the intestinal tracts removed. A sample of the body fluids was obtained by distillation. It contained 0.20 atom per cent deuterium. 12.21 gm. of total fatty acids (0.72 atom per cent deuterium) were obtained from the carcasses by the usual methods. The saturated acids were separated by precipitation of their lead salts. These were recrystallized to remove small amounts of unsaturated acids. They were then esterified with methyl alcohol and 4.4 gm. were used for fractional distillation. The result of the distillation are given in Table III.

Fraction 1 (m.p. 19.0-19.6°) must contain, besides palmitic acid, some of the lower fatty acids. Fraction 2 is still slightly contaminated with these, but Fractions 3 to 5 have the correct melting point of palmitic acid. For deuterium analysis Fractions 2 and 3 and Fractions 4 and 5 were combined. All contain a considerable amount of deuterium. The other Fractions 6, 7, and 8 and the residue contain large amounts of deuterium which must be due to the presence of unchanged deuterostearic acid of the diet.

The results of this distillation differ markedly from those of the artificial mixture given in Table II.

The presence of deuterium in the palmitic acid fractions cannot be taken as proof of its biological origin from stearic acid, since these fractions might have been contaminated with deuterostearic acid. This, however, is rather unlikely, since the deuterium content would require the presence of more than 10 per cent stearic acid (the deuterium content of the stearic acid must have been higher than 2.5 per cent).

In order to remove from the palmitic acid any contaminating deuterostearic acid, a large excess of ordinary deuterium-free methyl stearate was added to the combined Fractions 2, 3, 4, and 5. This mixture was again fractionated. The contaminating deuterostearic ester was diluted by the excess of normal stearic acid, from which it cannot be separated by fractional distillation, as their vapor pressures are practically identical. If all the deuterium in Fractions 2 to 5 were due to contamination with deuterostearic acid, the latter should represent about 12 per cent of the total. To 1.148 gm. of the combined Fractions 2 to 5 remaining after analysis were added 1.635 gm. of ordinary methyl stearate, an amount 11 times greater than the possible stearic ester contamination. It would thus be diluted 12 times. The results of the distillation are shown in Table IV.

The melting point of Fraction 1 is slightly depressed, obviously by the inclusion of the similarly contaminated Fraction 2 of Table III. Fractions 2 and 3 (Table IV), however, have the correct melting point of methyl palmitate. Fraction 3, after saponification and recrystallization, showed the correct melting point of palmitic acid. Fractions 4 to 8 are mixtures, but the residue consists of pure stearic acid, as shown by the melting points of both the methyl ester and the free acid. For deuterium analysis Fractions 2 and 3 and Fractions 7 and 8 were combined.

TABLE IV

Redistillation of Palmitic Acid from Mice Given Deuterostearic Acid

Fraction No.	Weight	Melting point of esters	Deuterium content
	mg.	*°C.*	*atom per cent*
1	241	27.0-27.8	0.32
2	181	29.0-29.3	0.32
3	164	29.1-29.4*	
4	336	25.8-26.4	0.23
5	200	29.0-31.8	0.13
6	141	34.0-34.5	0.09
7	413	37.0-37.3	0.05
8	123	36.6-37.0	
Residue	655	37.5-37.9†	0.05

* Melting point of free acid, 62.0-62.5°.

† Melting point of free acid, 68.5-69.5°.

In this second distillation, the deuterium content of the palmitic acid (0.32 atom per cent) is much greater than that of the stearic acid (0.05 atom per cent). The deuterium content of the palmitic acid fraction can therefore not possibly be due to contamination with stearic acid.

The water of the body fluids of the animals contained 0.20 atom per cent deuterium, originating from the breakdown of some of the deuterostearic acid given to the animals. This is much lower than that found in the palmitic acid (0.32 atom per cent). The body fluids in this experiment could therefore not have been the principal source of deuterium in the acid. The deuterium content of the palmitic acid is thus proof that it is derived from the conversion of stearic acid.

SUMMARY

1. Mice were fed for 5 days with deuterostearic acid and the palmitic acid was isolated from the carcasses. The deuterium content of the palmitic acid was proof that it had originated from the stearic acid.

2. Separation was carried out by fractional distillation of the methyl esters. A distillation apparatus is described for the separation of higher fatty acids from a mixture of 1 to 3 gm. The deuterium technique was used for testing the efficiency of the fractionating column.

3. A general method is discussed for the removal of a contaminating deutero substance from another one. It was employed for the removal of traces of deuterostearic acid from palmitic acid.

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BIBLIOGRAPHY

1. Channon, H. J., Jenkins, G. N., and Smith, J. A. B., *Biochem. J.*, **31**, 41 (1937).
2. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **117**, 485 (1937).
3. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **113**, 505 (1936).
4. Foote, P. D., Fairchild, C. O., and Harrison, T. R., *Bur. Standards Tech. Paper 170* (1921).

NOTES ON THE DETERMINATION OF SERUM INORGANIC PHOSPHATE AND SERUM PHOSPHATASE ACTIVITY

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The procedures for the determination of serum inorganic phosphate and of serum phosphatase, as published (1–4), have been in use in this and other laboratories for several years. We believe that a brief review of details of the procedure which have been discussed in personal communications and in the literature, and of certain other details where slight changes have yielded some advantage, is timely.

Serum versus Plasma—Aside from the lower phosphatase activity of oxalated plasma (4), the use of serum is justified by the relevance of serum calcium when inorganic serum phosphorus and serum phosphatase activity are determined. The serum may be kept in the refrigerator or in ice water for several hours after separation and centrifuging. If a longer delay is necessary, a small drop of toluene should be added. After 24 to 48 hours in the refrigerator the phosphatase activity is raised about 10 to 15 per cent (4); at room temperature (as in mailed specimens), the results are subject to an error of ±20 per cent. Clinically valid interpretations may, however, be based upon the analyses.

Substrate—The determination of phosphatase activity based on the liberation of inorganic phosphate from a substrate such as sodium β-glycerophosphate will remain justified, aside from other advantages of the substrate, by the following very important consideration: The difference between (*a*) serum inorganic phosphate and (*b*) total inorganic phosphate, after incubation of the serum with the substrate, is used as a measure of phosphatase activity; in addition, however, to such employment, the figure for serum inorganic phosphate is of independent importance in most, if not in all, cases where serum phosphatase determinations are required.

Solutions and Reagents—The use of 5 per cent trichloroacetic acid has been discontinued, and the dilute stannous chloride has been modified, with a simplification of calculations and manipulation, respectively. We investigated, and in some cases adopted for use, chemicals from other sources than those specified originally (the latter, it may be proper to add, had been satisfactory up to the time of the change, and may still be).

Blanks—In several cases (personal communications and a published report (5)) appreciable blanks were reported. It may be useful to repeat, therefore, that, with chemicals of the quality prescribed and reagents prepared according to our directions, the blanks are "at most faintly blue or green" (2). We had not estimated the color equivalent of the blank even approximately.

We now estimate the *maximum* color value of the blank under proper conditions as equivalent to about 0.0004 mg. of P. Even on the assumption that values twice as high would occur, the errors due to the blank would vary between about +2.5 per cent and −2 per cent, respectively, within the range of 0.0120 to 0.0360 mg. of P, the standard containing 0.020 mg. The corrections for the deviation from Beer's law being *3 to 4 times greater*, within the same range, the over-all correction, which *included* approximate correction for the blank, seems to have been justified.

Correction for Deviation from Beer's Law—The over-all corrections required seemed to be well fitted by a formula,

$$\text{Mg. P in aliquot} = \frac{0.48}{\text{reading of unknown}} - 0.0040$$

the standard containing 0.020 mg. and being set at 20 mm. For the values thus calculated and tabulated we claimed an accuracy of about 1 to 2 per cent *within the range of 0.0120 to 0.0360 mg.* and of about 3 to 5 per cent at the limits of the table's range, 0.0090 and 0.0560 mg. ((2) Table I).

We have used more recently a table based upon a formula which is applicable to a wider range of values of P (0.01 to 0.09 mg.): $\log (10{,}000a + 4) = 3.780 - 1.13 \log R$. This formula corresponds closely to a straight line indicated when the logs of known P values a are plotted against the logs of the corresponding color-

TABLE I

Inorganic P in Aliquot, at Stated Colorimetric Readings, Corrected for Deviation from Beer's Law. 0.02 Mg. Standard Set at 20 Mm.

Mm.......	0.0	0.2	0.4	0.6	0.8	D*	Mm.....	0.0	0.2	0.4	0.6	0.8	D*
mm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		*mm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
5	0 0974	0 0931	0 0892	0 0856	0 0823	18	21	0 01890	0 01869	0 01849	0 01829	0 01809	10
6	792	764	737	711	687	12	22	1790	1771	1753	1735	1717	9
7	665	645	626	607	588	9	23	1700	1683	1666	1650	1634	8
8	570	554	539	525	512	7	24	1618	1602	1587	1572	1557	8
9	499	487	475	464	453	6	25	1543	1529	1515	1501	1487	7
10	443	433	424	415	406	5	26	1474	1461	1448	1435	1422	7
11	398	390	382	374	367	4	27	1410	1398	1386	1374	1362	6
12	360	353	346	340	334	3	28	1351	1340	1329	1318	1307	6
13	328	322	316	311	306	3	29	1297	1287	1277	1267	1257	5
14	301	296	291	286	282	2	30	1247	1237	1228	1219	1210	5
15	278	274	270	266	262	2	31	1201	1192	1183	1174	1166	4
16	258	254	251	248	244	2	32	1158	1150	1142	1134	1126	4
17	241	238	235	232	229	1+	33	1118	1110	1102	1094	1087	4
18	226	223	220	217	215	1+	34	1080	1072	1065	1058	1051	4
19	212	209+	207	204+	202	1	35	1044	1037	1030	1023	1016	4
20	200	198−	195+	193	191	1	36	1010	1003	997	991	984	3

* In Column D are given the values for decrements corresponding to an increase of the readings by 0.1 mm., for use in interpolation.

imetric readings (*R*), and includes a correction for a blank value of 0.0004 mg.

Within the middle range, 0.0150 to 0.0500 mg., the calculated values (Table I) agree most closely with the older table ((2) Table I) and with known P values. In an upper range, 0.0500 to 0.0900 mg., errors do not exceed 2 or 3 per cent; within a lower range, 0.0150 to 0.0100 mg., the readings are more variable and the errors may reach 3 to 5 per cent.

Corrections for Color Inhibition—We have found it useful to introduce slight changes which have enabled us (*a*) to omit one reagent, (*b*) to simplify the correction factors for presence of trichloroacetic acid and glycerophosphate in the filtrates, and (*c*) to simplify calculations.

An extended series of comparisons indicated, within the limits of the error of the analytical method, that the color development in solutions of known P content was decreased equally by 9 per cent trichloroacetic acid and by our substrate, and that the effects were additive when both were present. It is conceivable that other substances—not introduced with the reagents, but present originally in serum—might have similar or contrary effects on color development. Our data, however, give no indication of such effects. The use of 10 per cent trichloroacetic acid in the preparation of *both* filtrates makes it possible to apply the *same* correction factor for the trichloroacetic acid in the "serum inorganic P filtrate," and for the trichloroacetic acid and glycerophosphate in the "total inorganic P filtrate." When high serum phosphatase activity indicates the desirability of a higher dilution of the filtrate than the standard dilution employed (20 times), correspondingly larger volumes of 10 per cent trichloroacetic acid may be used in precipitating the proteins after incubation of the serum with the substrate.

The correction for color inhibition amounts to between 1 and 1.5 per cent for each cc. of filtrate in the aliquot.

More rapid as well as more accurate calculation of the results is obtained by tabulating corrected values of P, in mg. per 100 cc., corresponding to given aliquots and colorimetric readings (see Table II).

Correction for Retardation of Hydrolysis—The standard conditions include "hydrolysis not exceeding 10 per cent of the substrate" (4). Within that limit the correction for retardation was omitted as being near the limits of accuracy of the method. This correction amounts to 0.1 per cent for each mg. of "total inorganic P" after incubation, per 100 cc. of serum. The correction is applied to the value of inorganic P liberated, per 100 cc. of serum, during the given period of incubation. If E represents the latter and Pt the former, then the corrected value of E, $Ec = E + Pt^2/1000$. In most cases serum inorganic P is so small a fraction of Pt that, if preferred, the following formula may be used (6): $Ec = E + E^2/1000$.

Aliquots—6, 4, and 2 cc. aliquots have been chosen, as covering well the usual range of phosphate values and permitting the use of half volumes. The use of smaller aliquots makes possible, in turn, the use of less serum, which is frequently desirable in clinical application as well as in experimental work with small animals.

Our present procedure follows.

Stock Solutions—Prepare, in accordance with earlier directions, the substrate, 10 per cent trichloroacetic acid, 7.5 per cent sodium molybdate solution (prepared from "N- and P-free" molybdic acid anhydride, Mallinckrodt's reagent quality), 10 N sulfuric acid (Mallinckrodt's reagent quality), 60 per cent stannous chloride solution (J. T. Baker, Analyzed), and the phosphate stock solution.

Phosphate Solutions—In addition to the phosphate standard solution (10 cc. of stock solution diluted to 300 cc.), which contains 0.02 mg. of P in 6 cc., prepare check solutions of known but arbitrarily varying concentration (0.5 to 3 or 4 times that of the standard) by suitable dilution of stock phosphate; preserve with toluene.

Reagents Prepared Daily—*Acid-molybdate solution.* To the *cold* 10 N sulfuric acid in a flask add, while mixing, an equal volume of 7.5 per cent sodium molybdate solution; when the mixture is properly prepared, it is free of even the slightest tinge of yellow. *Dilute stannous chloride solution.* Dilute 1 cc. of the 60 per cent solution to 400 cc. with water; *keep in a refrigerator between analyses.*

"Serum Inorganic P" Filtrate—To 1 cc. of serum in a test-tube add 9 cc. of 10 per cent trichloroacetic acid, mix well, and filter after a few minutes through 9 cm. filter paper, Whatman No. 44, or other "ashless" grade.

TABLE II

Inorganic P Content, in Mg. per 100 Cc., Corrected for Deviation from Beer's Law and for Effects of Trichloroacetic Acid and Glycerophosphate

The values are calculated for a dilution of 10 × (as in "inorganic P filtrate"). When the dilution is 20, 30, or 40 × (as in "total inorganic P" after incubation), these figures are to be multiplied by 2, 3, or 4, respectively. When 2 cc. aliquots are used, deduct 3 per cent from the value for the 4 cc. aliquot and multiply by 2.

	For 6 cc. aliquot (or for 3 cc. when half volumes are used)						For 4 cc. aliquots (or for 2 cc. when half volumes are used)					
Mm........	0.0	0.2	0.4	0.6	0.8	D*	0.0	0.2	0.4	0.6	0.8	D*
mm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
5	17.7	16.9	16.2	15.5	14.9	0.3+	25.8	24.7	23.7	22.7	21.8	0.5
6	14.4	13.8+	13.4	12.9	12.5	0.2+	20.9	20.1	19.4	18.8	18.2	0.3
7	12.0+	11.7	11.3	11.0	10.7	0.2−	17.6	17.1	16.6	16.1	15.6	0.2+
8	10.4	10.1	9.8	9.6−	9.3	0.1+	15.1	14.7	14.3	13.9	13.5	0.2
9	9.06	8.83	8.61	8.41	8.22	0.10	13.2	12.9	12.6	12.3	12.0	0.1+
10	8.04	7.87	7.70	7.54	7.38	0.08	11.7	11.5	11.2	11.0	10.7+	0.1+
11	7.23	7.09	6.95	6.81	6.67	0.07	10.5+	10.3	10.1	9.9	9.7	0.1
12	6.54	6.42	6.30	6.18	6.07	0.06	9.52	9.34	9.17	9.00	8.83	0.08+
13	5.96	5.86	5.76	5.66	5.56	0.05	8.67	8.51	8.36	8.22	8.09	0.07
14	5.47	5.38	5.29	5.21	5.13	0.04	7.96	7.83	7.71	7.59	7.47	0.06
15	5.05	4.97	4.90	4.83	4.76	0.04	7.35	7.24	7.13	7.02	6.92	0.05
16	4.69	4.62	4.56	4.50	4.44	0.03	6.82	6.72	6.63	6.54	6.45	0.05
17	4.38	4.32	4.26	4.20	4.15	0.03	6.36	6.28	6.20	6.12	6.04	0.04
18	4.10	4.05	4.00	3.95	3.90	0.02+	5.96	5.89	5.82	5.75	5.68	0.04
19	3.85	3.80	3.76	3.71	3.67	0.02	5.61	5.54	5.48	5.42	5.36	0.03
20	3.63	3.59	3.55	3.51	3.47	0.02	5.30	5.24	5.18	5.12	5.07	0.03
21	3.43	3.40	3.36	3.32	3.29	0.02−	5.02	4.96	4.91	4.86	4.81	0.03−

22	3 25	3 22	3 18	3 15	3 12	0 02−	4 76	4 71	4 66	4 61	4 56	0 03−
23	3 09	3 06	3 03	3 00	2 97	0 02−	4 52	4 47	4 42	4 38	4 34	0 02
24	2 94	2 91	2 88	2 86	2 83	0 01+	4 30	4 26	4 22	4 18	4 14	0 02
25	2 80	2 78−	2 75	2 73−	2 70	0 01+	4 10	4 06	4 02	3 98	3 94	0 02
26	2 68−	2 65	2 63	2 61−	2 58	0 01+	3 91	3 87	3 83	3 79	3 76	0 02
27	2 56	2 54	2 52−	2 50−	2 47+	0 01	3 73	3 69	3 66	3 63	3 60	0 02−
28	2 45	2 43	2 41	2 39	2 37+	0 01	3 57	3 54	3 51	3 48	3 45	0 01+
29	2 36−	2 34	2 32	2 30	2 28	0 01	3 43	3 40	3 38	3 35	3 33	0 01
30	2 27−	2 25−	2 23	2 21	2 20−	0 01	3 30	3 28	3 25	3 22	3 20	0 01
31	2 18	2 17−	2 15	2 13	2 12−	0 01	3 18	3 15	3 13	3 11	3 09	0 01
32	2 10	2 09	2 07	2 06	2 04		3 07	3 04	3 02	3 00	2 98	0 01
33	2 03	2 02	2 00	1 99	1 97		2 96	2 94	2 92	2 90	2 88	0 01
34	1 96	1 95	1 94	1 92	1 91		2 86	2 84	2 82	2 80	2 78	0 01
35	1 90	1 88	1 87	1 86	1 85		2 77	2 75	2 73	2 71	2 70	
36	1 84	1 82	1 81	1 80	1 79		2 68	2 66	2 64	2 63	2 61	

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*Columns D contain the values for decrements corresponding to an increase of the readings by 0.1 mm., for use in interpolation

"Total Inorganic P" Filtrate—Measure 10 cc. of substrate, preferably but not necessarily into a glass-stoppered test-tube (if half quantities are used, 1 cc. of petroleum ether precedes the substrate); place in a water bath at 37° for a few minutes, add 1 cc. of serum, with the tip of the pipette about 1 cm. above the surface of the liquid, invert once or tap to impart rotary motion, mixing the contents well (but without undue aeration); replace into water bath; remove after exactly 1 hour, cool in ice water for about 2 minutes, add 9 cc. of 10 per cent trichloroacetic acid (or more, if it is desirable to dilute more than 20 times), mix, let stand a few minutes, and filter as above.

It is desirable to use ½ or ¼ hour incubation periods in all cases in which high serum phosphatase activity is expected.

Reagent Blanks—Place 6 cc. of water in one test-tube, and 6 cc. of 10 per cent trichloroacetic acid in another; add to each 2 cc. of acid-molybdate solution and 2 cc. of dilute stannous chloride solution, mixing after each addition. The blanks should be colorless or at most tinged faintly green or blue.

Preparation of Aliquots for Analysis—The comparison standards (two or more in each set) contain 0.02 mg. of P in 6 cc. In accordance with the concentration expected, pipette 2, 4, or 6 cc. of filtrate, making up to a total volume of 6 cc. with water. Include in each set known check solutions, to verify the accuracy of the determination.

When colorimeter cups are employed of internal diameter of about 12 mm. or less, volumes of aliquots and of reagents (see below) may be halved.

Color Development and Comparison—To each tube, in sequence, add 2 cc. of acid-molybdate solution; mix by tapping, in the same sequence; add 2 cc. of dilute stannous chloride solution to each tube, mixing during the addition. The color of the mixture develops rapidly; colorimetric comparison may be made at convenience—immediately after the addition of stannous chloride or at any time within about 2 hours; we have found that a delay of ½ to 1 hour yields the best results.

Calculations—The P content of the check solutions may be obtained from Table I; the values of "serum inorganic P" and of "total inorganic P" after incubation, in mg. per 100 cc., are found in Table II, under the division corresponding to the volume of aliquot used. Multiply, if necessary, to allow for greater dilution than 10 × (see p. 172).

"Total inorganic P" *minus* "serum inorganic P" equals mg. of inorganic P liberated from glycerophosphate by 100 cc. of serum —or units of phosphatase activity per 100 cc., if the hydrolysis continued for 1 hour. If necessary, this value may be corrected for retardation of hydrolysis (see p. 170).

When $\frac{1}{2}$ or $\frac{1}{4}$ hour periods are employed for hydrolysis, multiply by 1.82 and 3.3, respectively; more rarely, when 2 or 3 hour periods are employed, the factors are 0.55 and 0.39, respectively (4).

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BIBLIOGRAPHY

1. Bodansky, A., Hallman, L. F., and Bonoff, R., *Proc. Soc. Exp. Biol. and Med.*, **28,** 762 (1931).
2. Bodansky, A., *J. Biol. Chem.*, **99,** 197 (1932–33).
3. Bodansky, A., *Proc. Soc. Exp. Biol. and Med.*, **28,** 760 (1931).
4. Bodansky, A., *J. Biol. Chem.*, **101,** 93 (1933).
5. Woodard, H. Q., Twombly, G. H., and Coley, B. L., *J. Clin. Inv.*, **15,** 193 (1936).
6. Bodansky, A., and Jaffe, H. L., *Am. J. Dis. Child.*, **48,** 1268 (1934).

THE REACTIONS OF NITRITE WITH HEMOGLOBIN DERIVATIVES

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There is perhaps no class of biological substances which has been so exhaustively studied as have the blood pigments. Starting with Mayow's (1674) demonstration that blood would yield a quantity of oxygen far in excess of that accountable on a basis of physical solution and the characterization by Hoppe-Seyler (1864) of hemoglobin as the oxygen-combining factor of blood, we have witnessed several stages in the study of the hemoglobin derivatives. Their engrossing spectroscopic pictures were exploited by the physicist and the ease with which most of them were crystallized with consequent assurance of their purity led the chemist to investigate their multiplicity of pathway in reaction. The biologist devoted his attention to the respiratory capacity of hemoglobin. Every division of the physical and biological sciences has lent to the study of hemoglobin its particular tools, and not only has the study in each division been exceedingly fruitful, but it is likewise true that the tools have emerged sharpened. Whereas now the spectrophotometer has replaced the naked eye of the earlier investigator and reactions involving oxidation and reduction can be followed potentiometrically, it must not be assumed that earlier work on hemoglobin should be discarded because it is thought to be crude. Owing to the fact that the earlier investigators were highly cognizant of the lability of the material with which they worked and because of an ingenuity which lack of adequate facilities forced on them, we find a surprising amount of scientific veracity buried in the literature published on the subject during the latter half of the 19th century. It is quite startling to the present day investigator to find that Hoppe-Seyler was the author of the prosthetic nucleus theory of the blood pigments; that he considered the iron in hemoglobin and the hemochromogens ferrous and that in methemoglobin and hematin as ferric. Many years before Bertin-Sans and de Motessier (1892) showed that pure hematin could not be reduced to hemochromogen, this same fact was demonstrated by Hoppe-Seyler. It would be well for every worker in the field to familiarize himself with the chapter on hemoglobin written by Gamgee (1898) in Schäfer's text-book, for in that chapter there is an astounding collection of fact and astute deduction. It is particularly of interest to note that Gamgee was the first to describe the interaction of nitrite with oxyhemoglobin and to classify the product as a methemoglobin derivative. Gamgee succeeded in crystallizing this nitrite hemoglobin, so called, though he found that the ratio of nitrite to hemoglobin was inconstant.

Kobert (1891), in his discussion of the nature of cyanmethemoglobin, claims to be the discoverer of a similar compound with the nitrites. Gamgee affirms that nitrite has no action upon reduced hemoglobin; but since, in the same sentence, he claims a similar inertness toward reduced hemoglobin of ferricyanide and of permanganate (both of which will immediately convert reduced hemoglobin to methemoglobin), the action of nitrite upon reduced hemoglobin bears reinvestigation.

Haurowitz (1924) classifies the reaction product of nitrite upon hemoglobin as a mixture of nitric oxide hemoglobin and methemoglobin. He disputes the conclusions of Hartridge (1920–21) who, unable to duplicate the spectroscopic picture of the reaction product by the superposition of the absorption bands of various pigments, claimed that nitrite methemoglobin was a definite compound.

Meier (1925) found that the reaction between nitrite and oxyhemoglobin varied with the hydrogen ion concentration of the solutions; that in acid solution methemoglobin resulted and in alkaline solution alkaline methemoglobin and nitric oxide hemoglobin were formed. Upon reduced hemoglobin, according to Meier, nitrite reacts to form methemoglobin and nitric oxide hemoglobin.

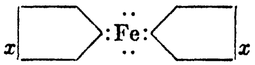
Aside from Hartridge, no investigator of a later period than Gamgee and Kobert postulates a nitrite hemoglobin.

Barnard (1932–33), in presenting electronic formulas of the prosthetic nucleus of methemoglobin, predicted its ability to combine with many electronegative radicals. A compound between methemoglobin and nitrite was mentioned specifically.

Within the last few years, a scheme of classification and nomenclature of the hemoglobin derivatives has been elaborated by the author. This classification has been withheld from publication because of the criticism that its introduction would burden an already confused literature. It becomes necessary at this point, however, to refer to the classification in order to understand the nature of nitrite hemoglobin for what subsequent research has shown it to be.

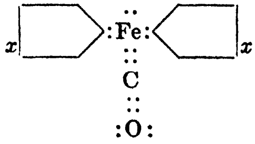
According to this scheme, the hemoglobin derivatives are classified with respect to: (1) the state of the iron which they contain; and (2) the nature of the extra iron linkages of the pyrrole groups.

Under (1) we recognize (*a*) the hemoferrons



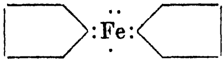
with ferrous iron, the latter having no free valences for electronic linkages. The auxilliary *x* may be absent, in which case it is reduced heme; or *x* may be present and consist of any nitrogenous base capable of carrying reduced heme into solution. Should *x* be undenaturated globin and the hemoferron be correctly associated with it, the compound is reduced hemoglobin.

(*b*) The hemoferryls

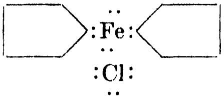


are polar valent compounds of hemoferron and oxygen-containing gases. That with carbon monoxide is best cited as an example. If *x* is absent, the formula represents carbon monoxide heme; if it be present and consist of the nitrogeneous bases mentioned under (*a*), carbon monoxide hemochromogen results.

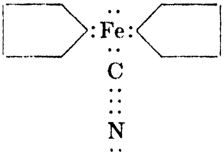
(*c*) The hemoferrins



contain iron in the ferric form. Pigments containing this prosthetic nucleus exhibit an absorption band in the red region of the visible spectrum and participate with hemoferron, as oxidant in an oxidation-reduction equilibrium. They may react with certain electronegative ions to form (*d*) the hemoferrides, which are salts of hemoferrin with acidic radicals. Thus



is hemin or hematin chloride (not hydrochloride) and



is a hemoferrin cyanide; hematin cyanide or cyanhematin if x is absent; and cyanmethemoglobin if x is undenatured globin.

Although this scheme has focused attention to the iron of the prosthetic nucleus, it must be understood that many of the properties of the pigments and their biological utility depend upon the the presence and nature of the attached auxiliary group. For instance, hemoferron owes its oxygenation capacity to a specific auxiliary globin (at least this is the one which occurs naturally, although there are unquestionably other nitrogenous substances which could be substituted and the prosthetic group rendered capable of reversible combination with oxygen). Modification of the globin fraction will usually affect the spectroscopic picture presented by the prosthetic nucleus as well as other physical and chemical properties of the substance as a whole.

According to this scheme, in either hemoferron or hemoferrin, there are at least three portals for chemical combination, one at the iron, the second in the porphyrin moiety, and the other at the point of attachment or in the nature of the auxiliary group. It will be shown that in the case of nitrite, both combination with the iron of the hemoferrins and modification of the auxiliary group, when this is globin, take place. The result is a compound, the nature of which has not heretofore been exactly delineated, though it has been approximated when classified as nitrite methemoglobin.

That nitrite will combine with the ferric iron of methemoglobin was considered to be in harmony with the electronic structure of hemoferrin which combines with electronegative ions. As examples of such electronegative ions, we may cite cyanide ion, hydroxyl ion and, according to the work of Keilin and Hartree (1935), peroxide ion, sulfide ion (Keilin, 1933), and azide ion (Smith and Wolf, 1904).

In all these instances of combination, there is a change in the spectroscopic picture of the methemoglobin, this invariably consisting, in part, of a disappearance of the band in the red region. Therefore we should expect that, if nitrite combined with methemoglobin to form a compound of similar nature to the ones cited above, the addition of nitrite to methemoglobin would cause a disappearance of the red band evinced by the latter. This actually takes place, as is indicated in the spectrophotometric absorption curve of nitrited methemoglobin. But such a disappearance of the absorption band in the red might likewise be explained on a basis of the reduction of ferric hemoglobin to a ferrous hemoglobin derivative. The latter is unlikely. Nitrite oxidizes oxyhemoglobin to methemoglobin, as shown by Barcroft and Müller (1911–12). The possibility that it can likewise reduce methemoglobin to a ferrous hemoglobin is therefore highly remote. In this connection, the experiment of Meier (1925), conducted by adding nitrite to an oxyhemoglobin solution in increasing ratios, is of interest. In acid solution (pH 5), where the red band of methemoglobin is of course most intense, the addition of 1 M equivalent of nitrite to oxyhemoglobin led to the appearance of a strong band in the red (methemoglobin formation). Continued addition of nitrite, however, led to a fading of this band. At the next ratio given in Meier's table (nitrite to hemoglobin = 5:1), the color had changed from brown to brownish red. At a nitrite ratio of 100:1, the spectrum becomes "uncharacteristic." These findings have been confirmed in this investigation and the disappearance of the band in the red is inferred to mean an inhibition of the ionization of hemoferrin, which could take place only through combination.

EXPERIMENTAL

That nitrite will combine with hemoferrin and not with hemoferron was demonstrated electrometrically by the effect of nitrite on the potentials developed by poised solutions containing hemoglobin and methemoglobin. The potentials in the presence of nitrite are not well defined but, as can be seen from Table I, they are shifted to the negative side. Characterizing the potentials developed in the methemoglobin-hemoglobin system, we have the equation

$$e_0 = e_1 - \frac{RT}{nF} \log_e \frac{(\text{Met})}{(\text{Hb})}$$

and transposing

$$e_1 = \frac{RT}{nF} \log_e \frac{(\text{Met})}{(\text{Hb})} - e_0$$

The observed potential therefore will vary logarithmically with the relative effective concentration of methemoglobin, as oxidant to hemoglobin. A shift in the negative direction—on the addition of nitrite—indicates either that the effective oxidant concentration of methemoglobin is reduced or that the system is evincing the reduction potential of nitrite ion. The latter possibility is rendered untenable by the fact that when ferricyanide is added, it evinces its oxidation potential after a sufficient addition to oxidize the reduced hemoglobin present but before sufficient has been added to oxidize the large excess of nitrite present. This experiment further indicates that the hemoferron nucleus is unaltered by the nitrite, since it retains its reductant position before oxidation by the ferricyanide. There is, of course, the possibility of combination between hemoferron and nitrite with a retention of the reductant activity of the former, but this behavior would be unparalleled in the other combinations of hemoferron. We are therefore justified in concluding that in the presence of nitrite the oxidant concentration of methemoglobin is diminished by the formation of an undissociated nitrite compound: $(\text{Met}^+) + (\text{NO}_2^-) = (\text{MetNO}_2)$.

But, if nitrite combines with the hemoferrin of methemoglobin, it should likewise with that of hematin. Fig. 1 shows the spectrophotometric absorption curves obtained with methemoglobin and with hematin in the presence of nitrite. The similarity between the two curves is striking, yet neither shows the band in the red that is considered characteristic of what we term hemoferrin. Any band, if it exists at all, would seem to be shifted toward the violet, as is seen in other hemoferrides.

The similarity of the absorption spectra of hematin and hemoglobin in nitrite solution is further brought out by a consideration of Table II in which the percentage transmissions of these substances at each wave-length are compared with a solution of hemoglobin in nitrite. Obviously in comparing two solutions of hemoglobin in nitrite (as was done to secure the figures in the right-hand section of Table II) the transmission percentages will be 100 at all wave-lengths. Likewise, in comparing hematin in nitrite against the former, the percentage transmission at all wave-lengths is as close to 100 as spectrophotometric accuracy will permit.

TABLE I

Effect of Nitrite on Oxidation-Reduction Potential of Methemoglobin-Hemoglobin System

$NaNO_2$	$K_3Fe(CN)_6$	Hb	MetHb	e_1
mM	*mM*	*mM*	*mM*	
0.00		0.035		−0.208
0.04		0.035		−0.251
0.08		0.035		−0.234
0.14		0.035		−0.227
0.42		0.035		−0.247
0.60		0.035		−0.237
0.71		0.035		−0.246
0.94		0.035		−0.252
0.94	0.004	0.031	0.004	−0.206
0.94	0.008	0.027	0.008	−0.129
0.94	0.020	0.015	0.020	0.043
0.94	0.024	0.011	0.024	0.083
0.94	0.032	0.003	0.032	0.129

In the above spectrophotometric study, the hematin was prepared by denaturation of oxyhemoglobin with sufficient NaOH solution to carry the pH to 10.7. Then the solution was brought back to about pH 7.6 with HCl solution, by electrometric titration with the hydrogen electrode. The hematin was unquestionably combined with globin (cathemoglobin). Addition of nitrite to pure alkaline hematin solutions would not be valid, since alkaline hematin is probably a hemoferride, hemoferrin hydroxide. Should nitrite be added to such a solution of hematin, there would then be competition for the free iron valence between hydroxyl ion and nitrite ion, and as yet we have no information as to the relative dissociation constants of hematin hydroxide and hematin nitrite.

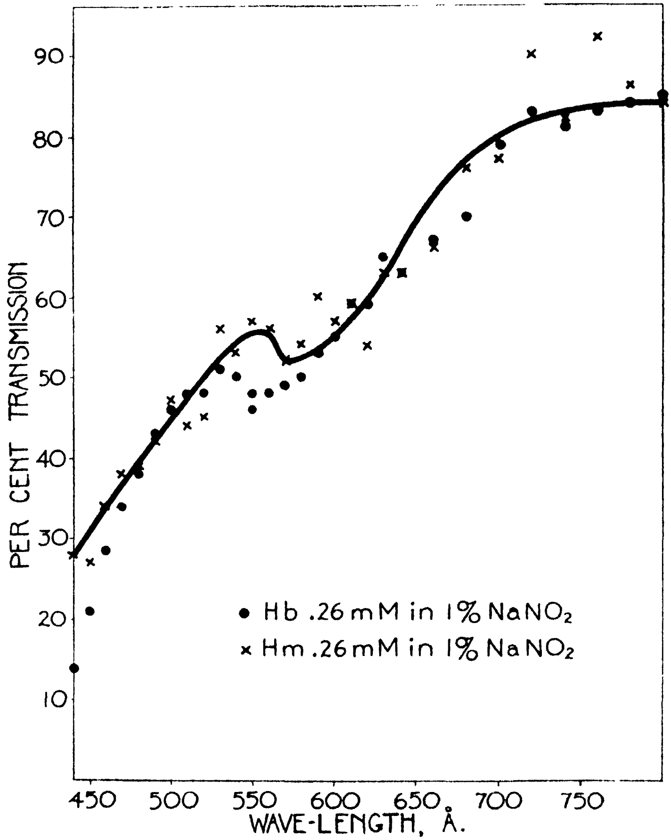


Fig. 1. Absorption curves obtained with methemoglobin and with hematin in the presence of nitrite.

When hematin is dissolved in aqua ammonia, however, it probably exists as hemoferrin. Likewise, in concentrated ammonia water, hematin is soluble and has a reddish color, grossly different from that of its solutions in the carbonates or hydroxides of the alkali metals. From its solution in ammonium hydroxide, hematin is precipitated by the addition of sodium nitrite.

0.5 cc. of 10 per cent $NaNO_2$ solution was added to 20 cc. of a 1 per cent solution of hematin in concentrated ammonium hydroxide solution. There was an immediate precipitation of all the coloring matter, and the extraction of this reaction product, which was green and amorphous, with 5 per cent acetic acid yielded nitrous acid.

TABLE II

Spectrophotometric Transmission Ratios of Hematin in Nitrite to Methemoglobin in Nitrite at Different Wave-Lengths

λ Å.	Hm/Hb			Hb/Hb*		
	Original	Reversed	Average	Original	Reversed	Average
460	60				95	
480	69	77	73	78	93	86
500	81	97	89	83	98	90.5
520	84	99	91.5	94	89	91.5
540	97	104	100 5	91	110	100.5
560	91	110	100.5	87	104	90.5
580	84	99	91 5	92	104	98
600	80	102	91	90	106	98
620	84	99	96 5	85	100	92.5
640	97	103	100	88	104	96
660	91	101	96	85	106	95.5
680	87	101	94	85	110	97.5
700	87	102	94 5	97	109	103
720	89	103	96	89	106	97.5

* Both chambers contain hemoglobin in nitrite solution. There is therefore a comparison of identical solutions and the average percentage transmission at all wave-lengths should be 100 per cent within spectrophotometric error.

In this case we have combination between a prosthetic radical and nitrite. The recovery of unaltered nitrous acid would adjudicate the type of union to the class of salt formation, for, although hematin may contain primary and secondary amino groups with which nitrite (as nitrous acid) might react, if such were the case it should not be recovered after reaction. (With primary amines, nitrogen is evolved and with secondary amines a nitrosamine results.) We cannot use the argument that in alkaline solutions of the pH of concentrated ammonium hydroxide, so little nitrous acid exists as to render such reaction impossible, for, as will be shown, nitrites do react with globin at a pH above neutrality. But is the salt formation, in the case of combination between hematin and nitrites, with the iron? Thus far, all the evidence presented has been indirect. It is so assumed from the alteration in the oxidant nature of methemoglobin and the shift in the absorption band of both methemoglobin and hematin. It was therefore decided to test the effect of nitrite on hemoglobin, in which substance the hemoferrin iron is absent.

100 cc. of a 0.05 mm solution of reduced hemoglobin were treated with 2 cc. of a 2 per cent solution of sodium nitrite which had been aerated with nitrogen to wash out dissolved oxygen. Within a minute, the reaction mixture had turned cherry-red and, when subjected to spectroscopic examination, showed the absorption doublet characteristic of hemochromogen. This shows that the globin is the seat of modification in the reaction of nitrite on reduced hemoglobin; since hemochromogen is composed of the hemoferron radical and denaturated globin, the prosthetic nucleus must remain unaltered but the globin must be modified by nitrite. This alteration presumably obtains in the globin linked to methemoglobin or oxyhemoglobin when nitrite is added to their solutions and this furnishes an explanation of the conversion of oxyhemoglobin into what has been termed methemoglobin but is actually a cathemoglobin. Since oxygen will immediately oxidize the hemoferron radical unless the latter be poised or stabilized by attachment to undenaturated globin, when the nitrite reacts with the globin, it renders the latter unsuitable for such stabilization of the oxygen-combining power of hemoferron.

Action of Nitrites on Blood Pigment in Acid Solution

If to about 10 cc. of 2 per cent $NaNO_2$ solution in a test-tube a drop of concentrated hydrochloric acid or lactic acid be added, and, while the solution is still effervescing, a few drops of a dilute (about 1 mm) solution of oxyhemoglobin, methemoglobin, or nitrite hemoglobin be added, a cherry-red color with a violet nuance immediately develops. This cherry-red color persists for only a few seconds and turns to a brownish red with greenish fluorescence. At this time the bands of hematoporphyrin appear.

The cherry-red pigment is assumed to be a hemochromogen, although it is not sufficiently stable for a positive identification of its absorption bands. That it is a hemochromogen is inferred from the ease with which it transforms to porphyrin. The ease of the removal of iron from hemochromogen by weak acids has been pointed out by Gamgee (1898) and the reduction of the blood pigment to the hemoferron state could be expected of nitrous acid; the denaturation of globin is also in keeping with its behavior.

Since it was desired to study further the cherry-red pigment obtained from nitrite hemoglobin by the action of acid, recourse was had to the weak dissociation of organic acids in ether solution, a menstruum in which heme is likewise soluble.

A solution of hematin in acid ether was prepared by extracting dried blood with boiling glacial acetic acid for 1 hour under a reflux condenser. The pigment concentration was brought to 1 mM by the addition of more glacial acetic acid. An equal volume of ether was added and the precipitated protein filtered off. This solution shows a strong absorption band in the red region of the visible spectrum, centering at 620 Å. When a drop of 10 per cent sodium nitrite is added to 5 cc. of this solution in a spectroscopic observation vessel, effervescence takes place and the solution becomes cherry-red, the band in the red disappears to be replaced by a doublet in the yellow-green, a thin band centering at 565 Å., and a band about twice as wide as the former, centering at 522 Å.

These bands fade with the cessation of the effervescence, so that plotting of a spectrophotometric absorption curve is out of the question. In two samples, the limits of the band in the green were 513 to 530 Å. and 514 to 530 Å. The band in the yellow extended from 558 to 572 Å. in the first and from 560 to 572.5 Å. in the second sample. After the bands have faded, the solution has a more yellow cast than the original hematin solution. It shows no definite bands in the visible spectrum but the further addition of nitrite causes the reappearance of the doublet described above. This in turn likewise fades with the decomposition of the nitrite and appears to be restorable practically indefinitely simply by addition of more nitrite. With the fading of the doublet there is no return of the band in the red, so that the original substance must have undergone modification.

The saturation of the cherry-red pigment with carbon monoxide causes no change in its bands.

What is the nature of the substance? It is unquestionably a hemochromogen, possibly a nitric oxide hemochromogen. Nitrites in acid solution are powerful reducing agents and nitric oxide is evolved from such solutions. But after the disappearance of the hemochromogen (should this actually be the nature of the reddish pigment) the original hematin is not reformed, as judged from the fact that the absorption band in the red is no longer present. Apparently nitrous acid reacts with some other portion of the hematin molecule and suspicion is strongly directed to the pyrrole nitrogen. This is not as yet demonstrated. That some reaction at another portion of the hematin molecule, other than the iron, does take place, under the influence of nitrite, with a modification of the properties of hematin, is illustrated by the results of the following experiment.

20 cc. of a 0.5 mm solution of crystalline hemin in concentrated ammonium hydroxide solution is separated into two portions, one of which is treated with 0.1 gm. of sodium cyanide to convert the hematin to cyanhematin. On addition of 1 cc. of 10 per cent sodium nitrite solution to each sample, the hematin in solution is precipitated out as a greenish substance, as described in an experiment mentioned above. The cyanhematin in solution is likewise precipitated by this procedure, but it retains its orange color in becoming insoluble. The nitrite has been unable to replace the cyanide at its iron linkage, but it has modified the compound so that its solubility in ammonium hydroxide is vitiated.

We must therefore postulate that in its reaction with nitrite the methemoglobin molecule may be modified in three distinct manners: (1) reaction of nitrite with the globin and the denaturation of the latter, (2) compound formation between the ferric iron and nitrite ion, and (3) reaction with the heme radical aside from the iron.

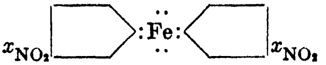
DISCUSSION

These results are entirely in harmony with those reported by Hartridge (1920–21). He determined the effect of nitrite on a hemoferryl, carbon monoxide hemoglobin, and, likewise, in his experiments the nitrite was without effect on the prosthetic group. He assumed that nitrite had no effect upon carbon monoxide hemoglobin, since its absorption bands persisted after treatment by nitrite and he rightfully claimed that it was only methemoglobin with which nitrite combined. This is true as long as we confine ourselves to a discussion of compound formation with the iron of these compounds. We must assume, however, that the effect of nitrite on the globin of carbon monoxide hemoglobin is the same as that on the globin of methemoglobin, a denaturation which would lead to the formation of carbon monoxide hemochromogen. The absorption bands of the substance last mentioned are identical with those of carbon monoxide hemoglobin, as is agreed by all investigators and as is likewise demonstrated in this study.

A confirmation of the work of Hartridge (1920–21) on the persistence of the bands of carbon monoxide hemoglobin was obtained by comparing the bands of a 0.5 mm solution of the substance in the upper chamber of a spectrophotometer vessel with an identical solution made up in 1 per cent $NaNO_2$ solution in the lower chamber. The double vessel is placed before the collimator of a Hilger spectrophotometer so that each solution casts corresponding spectra and, on widening the observing slit, one may observe the exact identity of the bands. After 2 days, the bands still coincide, though the nitrited carbon monoxide hemoglobin shows a gross change in color.

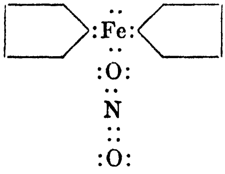
The coincidence of the absorption bands of carbon monoxide hemoglobin and carbon monoxide hemochromogen can be demonstrated if the solution in the lower chamber be treated with hydrazine hydroxide (to a total concentration of 4 per cent) instead of with nitrite as before. Hydrazine hydroxide immediately converts carbon monoxide hemoglobin to the corresponding hydrazine hemochromogen, but the bands of both substances continue to coincide for several hours, those of the hemochromogen gradually fading as the solution becomes colorless.

Any combination between nitrite and hemoferron would be at variance with the theory previously presented. Scrutiny of the hemoferron nucleus shows no place for combination with the iron, though combination with the pyrrole or the auxiliary group is theoretically possible. That the latter reacts with nitrite is shown by the transformation of reduced hemoglobin to hemochromogen. The reaction product of reduced hemoglobin and nitrites can be represented by



where x_{NO_2} represents globin as modified by nitrite.

In the case of those hemoferryls and hemoferrins which have globin auxiliaries, this same modification of the globin under the influence of nitrite unquestionably takes place. In addition, in the hemoferrins combination can also take place at the iron, so that the reaction product between nitrite and methemoglobin (which should be called nitrite cathemoglobin and not nitrite methemoglobin, since the globin is no longer natural globin) may be represented as



that is, with nitrite electronically combined with the iron and having modified the globin.

The ratio of the nitrite molarity of that nitrite attached to the iron to the iron molarity would be 1, but this ratio would be masked on analysis by the amount reacting with the globin fraction, an amount which would depend on many as yet unknown factors and apt to be (as Gamgee indeed found it) inconstant. It must also be remembered that globin is an ampholyte that can bind cations such as nitrite ion.

SUMMARY

A classification and nomenclature for the hemoglobin derivatives, based on the state of the contained iron, is submitted.

Potentiometric observation shows that nitrite combines with the prosthetic nucleus iron of methemoglobin and diminishes its oxidant activity.

Spectrophotometric absorption curves of methemoglobin in nitrite solution and hematin in nitrite solution show either an identity or close similarity of their respective reaction products.

Nitrite does not combine with the prosthetic nucleus iron of the ferrous hemoglobin derivatives.

Nitrite combines with hematin in ammonium hydroxide solution to form a dissociable compound.

Nitrite reacts with the globin of hemoglobin derivatives. The nature of this reaction has not been determined, but the globin is denaturated.

Nitrous acid reduces hematin in acid ether to a compound similar to hemochromogen.

Nitrite hemoglobin, commonly termed, which results from the reaction of nitrite on methemoglobin or oxyhemoglobin, is a nitrite cathemoglobin.

BIBLIOGRAPHY

Barcroft, J., and Müller, F., *J. Physiol.*, **43**, xx (1911–12).

Barnard, R. D., *J. Gen. Physiol.*, **16**, 657 (1932–33).

Bertin-Sans, H., and de Motessier, J., *Compt. rend. Acad.*, **114**, 923 (1892).

Gamgee, A., in Schäfer, E. A., A text-book of physiology, Edinburgh, 242 (1898).

Hartridge, H., *J. Physiol.*, **54**, 253 (1920–21).

Haurowitz, F., *Z. physiol. Chem.*, **138**, 68 (1924).

Hoppe-Seyler, F., (1864) cited by Gamgee.

Keilin, D., *Proc. Royal Soc. London, Series B*, **113**, 393 (1933).

Keilin, D., and Hartree, E. F., *Proc. Royal Soc. London, Series B*, **117**, 1 (1935).

Kobert, F., (1891) cited by Gamgee.

Mayow, J., Tractatus quinti, Oxonii (1674).

Meier, R., *Arch. exp. Path. u. Pharmakol.*, **110**, 241 (1925).

Smith, L., and Wolf, C. G. L., *J. Med. Research*, **12**, 451 (1904).

RESYNTHESIS OF MUSCLE GLYCOGEN FROM HEXOSEMONOPHOSPHATE

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In 1920 Meyerhof (1) published six experiments in which he demonstrated a resynthesis of glycogen in isolated frog muscle. The experimental procedure and results were as follows: Hind legs of frogs were stimulated to fatigue by single shocks. One hind leg was then analyzed for glycogen, intermediary carbohydrates, and lactic acid, while the other was kept in O_2 at 14° for 21 to 24 hours before being analyzed in the same manner as its mate. The gastrocnemius served for the measurement of respiration. About 24 hours were required for a return of the O_2 consumption of the stimulated muscle to the resting level. This long period of recovery was due to the thickness of the muscles used and to the high rate of respiration prevailing after the stimulation, making the rate of diffusion of O_2 insufficient for a considerable length of time. On an average, the lactic acid decreased 256 mg. per cent, while the total O_2 consumption during the recovery period corresponded to the oxidation of only 130 mg. per cent of lactic acid, leaving 126 mg. per cent of lactic acid to be accounted for. The observed increase in glycogen was 118 mg. per cent. The extra O_2 consumption (total minus resting O_2 consumption) amounted to 70 mg. in terms of lactic acid, while 256 mg. of lactic acid disappeared; hence the oxidative quotient was $256/70 = 3.66$.

It was shown later by Meyerhof, Lohmann, and Meyer (2) that it makes no difference whether the lactic acid is formed in the muscle itself as the result of activity or whether the lactic acid diffuses into the muscle from the outside (by immersing sartorii in Ringer's solution containing lactate); in both cases glycogen is formed at the expense of the disappearing lactate, the naturally occurring *l*(+)-lactic acid being much more active than the *d*(−)-lactic acid. The resynthesis of glycogen in previously stimulated frog muscle has been confirmed by Foster and Moyle (3) and Hahn, Fischbach, and Niemer (4). In numerous other experiments on muscle, a summary of which is given by Lohmann (5), the resynthesis of glycogen was not demonstrated by direct analysis, but was deduced indirectly from the oxidative quotient, *i.e.* from a greater disappearance of lactate than could be accounted for by the extra O_2 consumption.

Lactic acid is not the only product of glycogen breakdown which accumulates in a contracting muscle and disappears during the recovery period. As shown previously (6), as much as 50 per cent of the glycogen broken down during contraction may be present in muscle as hexosemonophosphate, the exact amount depending on the method of stimulation. During recovery the phosphate ester disappears at about the same rate as lactic acid. The present experiments were undertaken in order to determine the fate of the hexosemonophosphate which disappears during the recovery process of muscle.

EXPERIMENTAL

First Series of Experiments[1]—Muscles (sartorius, ileofibularis, semitendinosus, tibialis) of small specimens of *Rana pipiens* were used. The four muscles weighed between 250 and 350 mg., so that the recovery period could be as short as 5 hours. In order to obtain enough material for analysis, the muscles of three frogs were combined in each experiment. After spinal transection, both hind legs were tetanized simultaneously through the pelvic nerves (three times for 10 seconds with 10 second pauses between the stimulations). The muscles were dissected on an ice-cold plate with as little damage as possible, and those of one side were analyzed immediately. The matched muscles of the other side, after being weighed, were hung in a moist chamber[2] through which a slow stream of O_2 was passing and were kept for 5 hours at 15°, when they were analyzed in the same way as their mates.

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[1] We are indebted to Dr. John O. Closs for part of the analytical work in connection with these experiments.

[2] Unless the chamber is completely saturated with moisture, the muscles may lose considerable weight owing to drying. With complete saturation the muscles gained slightly in weight during the recovery period.

The muscles were frozen in CO_2 snow and powdered in an apparatus which was, except for its size, similar to that described by Graeser, Ginsberg, and Friedemann (7). The powdered mass was transferred quantitatively to a mortar containing 15 cc. of 0.5 N HCl and was triturated with a pestle. After centrifugation, the supernatant fluid was poured off and measured in a calibrated centrifuge tube. Glycogen was determined in the residue and in an aliquot part of the supernatant fluid after digestion with 30 per cent KOH and precipitation with boiling alcohol (8). The rest of the supernatant fluid was deproteinized by addition of solid $HgCl_2$ and was used for lactic acid (9) and hexosemonophosphate (10) determinations.[3]

Since only small changes in glycogen could be expected, it was necessary to use frogs with less than 1 per cent of muscle glycogen. Frogs kept in the laboratory without food until early summer answered this purpose. In fresh shipments of frogs the muscle glycogen was generally too high for this type of experiment.

Control experiments were carried out which showed that closely agreeing values were obtained when matched muscles of the right and left sides were compared immediately after the stimulation in regard to their glycogen and lactic acid content. In seven different frogs the glycogen levels (in mg. per cent) were 411, 521, 552, 759, 570, 955, 593, *average 623*, in the small muscles of the right side and 401, 536, 567, 805, 540, 915, 620, *average 626*, in the corresponding muscles of the left side. Similarly, four experiments showed lactic acid levels (in mg. per cent) of 165, 162, 160, 161, *average 162*, and 168, 167, 151, 157, *average 160*, in matched muscles of the right and left sides, respectively.

The following values for the O_2 consumption at 15° were obtained on pairs of unstimulated muscles taken from four different frogs: 25.2, 25.1; 42.3, 38.4; 38.0, 31.6; 38.8, 39.0; average 34.8 mg. per 100 gm. per 5 hours, corresponding to the oxidation of 33 mg. of carbohydrate. In four experiments the O_2 consumption of previously stimulated muscle amounted to 60 to 81 mg. of O_2 per 100 gm. of muscle during the 5 hour period of recovery, corresponding to 56 to 76 mg. of carbohydrate oxidized. (In both cases it was assumed that the respiratory quotient was unity.)

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[3] Previous experiments (11) have shown that maltose or other complex sugars do not accumulate during tetanic stimulation of muscle. For this reason it appeared unnecessary to determine "intermediary carbohydrates" as had been done by Meyerhof.

The experiments in Table I show that the glycogen increased in each case when previously stimulated muscle was kept for 5 hours in O_2. On an average, the lactic acid decreased 98 and the hexosemonophosphate 72 mg. per cent, as compared to a gain in glycogen of 97 mg. per cent. The difference between carbohydrate lost and gained, namely $170 - 97 = 73$ mg. per cent, probably represents carbohydrate oxidized, a figure which is of the same order of magnitude as that actually determined in the respiration experiments cited in the preceding paragraph. In order to calculate the oxidative quotient, it is necessary to deduct the basal from the total O_2 consumption; this gives an extra O_2 consumption (in terms of carbohydrate oxidized) of $73 - 33 = 40$ mg. per 100 gm. of muscle per 5 hours. The total amount of carbohydrate which disappears (*i.e.* lactic acid plus hexosemonophosphate) gives a quotient of $170/40 = 4.2$, but, if the disappearance of lactic acid alone is considered, the quotient would be below that usually found, namely $98/40 = 2.4$.

TABLE I

Aerobic Resynthesis of Glycogen in Frog Muscle

The period of aerobic recovery was 5 hours at 15°. All values are given in mg. per 100 gm. of muscle.

After stimulation			After recovery in O_2			Change in		
Lactic acid	Hexosemonophosphate (as hexose)	Glycogen	Lactic acid	Hexosemonophosphate (as hexose)	Glycogen	Lactic acid	Hexosemonophosphate (as hexose)	Glycogen
157	122	431	61	38	522	−96	−84	+91
159	154	960	78	66	1040	−81	−88	+80
200	111	510	91	55	644	−109	−56	+134
192	107	498	119	76	553	−73	−31	+55
198	130	965	98	61	1052	−100	−69	+87
202	156	698	74	53	834	−128	−103	+136
Average						−98	−72	+97

The formation and disappearance of hexosemonophosphate were not determined in Meyerhof's experiments; the occurrence of this ester in muscle was not known at that time. A few experiments were carried out with the form of stimulation used by Meyerhof, namely single shocks at the rate of 60 per minute to complete fatigue. The hexosemonophosphate levels immediately after stimulation were 60, 40, and 53 mg. per cent, and the disappearance during 5 hours of recovery amounted to 28, 3, and 8 mg. per cent, respectively. The accumulation of lactic acid in a muscle fatigued by single shocks was much greater than in one subjected to three 10 second tetani. The changes in hexosemonophosphate in Meyerhof's experiments were therefore quantitatively much less important than in those here reported.

The effect of temperature on the increase in glycogen during the recovery period of previously stimulated muscle was investigated. At 20° a smaller increase in glycogen was found than at 15°. Four experiments at 20° showed an increase in glycogen of 25, 105, 53, 49, average 53 mg. per cent, as compared to an increase of 97 mg. per cent in the experiments in Table I in which the recovery took place at 15°. At 25–27° more carbohydrate was oxidized, owing to the greatly increased O_2 consumption, than was resynthesized, so that the glycogen balance sometimes became negative. In five experiments the glycogen changes were +18, −63, −24, +16, −18, average −14 mg. per cent.

Second Series of Experiments—In order to arrive at a more definite conclusion as to the fate of hexosemonophosphate during the recovery process of muscle, it was necessary to devise experiments in which, prior to recovery, the muscles contained more hexosemonophosphate than lactic acid. The following method was adopted. A few hours prior to the experiments frogs were pithed in such a way that the hind legs were immobilized but that respiration and circulation remained intact. The hind legs were then tetanized three times for 10 seconds by applying an electrode to the lumbar region after removal of the skin. The circulation was left intact for 5 minutes after the stimulation. In this way a considerable amount of lactic acid was removed, while the hexosemonophosphate which does not diffuse out remained at a high level. Since very small muscles were used, the recovery period in oxygen was reduced to 2 hours. Changes in glycogen, lactic acid, and hexosemonophosphate were determined in separate series of experiments.

TABLE II

Restitution of Muscle Glycogen during 2 Hours of Aerobic Recovery

The temperature was 15°. The figures express mg. of glycogen per 100 gm. of muscle.

After stimulation	After recovery in O_2	Change in glycogen
483	608	+125
1410	1520	+110
(567)	(507)	(−60)
1561	1715	+154
893	968	+75
739	805	+66
482	559	+77
381	423	+42
501	572	+71
Average. .806	896	+90

The values for the negative experiment (given in parentheses) are not included in the averages.

TABLE III

Disappearance of Lactic Acid during 2 Hours of Aerobic Recovery

The temperature was 15°. The figures express mg. of lactic acid per 100 gm. of muscle.

After stimulation	After recovery in O_2	Change in lactic acid
102	46	−56
107	53	−54
76	35	−41
85	58	−27
92	62	−30
67	36	−31
86	55	−31
93	59	−34
Average. .88	50	−38

Control experiments consisted in measuring glycogen on the right and left side after stimulation. The differences were +40,

+24, −26, +30, −11, −38, average +3 mg. per cent, in favor of one side. Table II shows that in eight out of nine experiments the glycogen content of muscles increased during 2 hours of aerobic restoration, the average being +90 mg. per cent of glycogen in favor of the restored muscle, when the one negative experiment is excluded. The average amount of lactic acid which disappeared during 2 hours of aerobic recovery was only 38 mg. per cent (Table III). Hence, at least 90 − 38 = 52 mg. per cent of glycogen must have been formed from sources other than lactic acid. (This calculation does not take into account that one-fourth of the lactic acid which disappeared might have been oxidized.) In Table IV (first three columns) the average amount of hexosemonophosphate which disappeared during 1 to 1.5[4] hours of aerobic restoration was of the same order of magnitude as the calculated gain in glycogen from sources other than lactic acid (−50 as compared to +52 mg. per cent). It seems probable, therefore, that a large

TABLE IV

Disappearance of Hexosemonophosphate in Frog Muscle without and with Iodoacetate Poisoning

The period of recovery in O_2 was 1 to 1.5 hours at 15°. The figures express mg. of hexosemonophosphate (as hexose) per 100 gm. of muscle.

Without iodoacetate			With iodoacetate		
After stimulation	After recovery in O_2	Change in hexosemonophosphate	After stimulation	After recovery in O_2	Change in hexosemonophosphate
123	48	−75	135	75	−60
88	71	−17	114	86	−28
157	110	−47	146	105	−41
161	112	−49	129	100	−29
101	39	−62	143	64	−79
			138	87	−51
			151	105	−46
Average....		−50			−48

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[4] A shorter period of aerobic recovery was chosen because of the necessity of avoiding the development of rigor in the experiments with iodoacetate. During rigor there occurs a marked increase in hexosemonophosphate.

part of the hexosemonophosphate which disappears aerobically is reconverted to glycogen.

The poisoning with iodoacetate (Table IV) was accomplished in the following manner. The hind legs of previously pithed frogs were tetanized through the exposed sciatic nerves. Immediately after the stimulation 10 mg. of neutralized iodoacetic acid were injected intravenously. The gastrocnemii, removed 5 minutes after the injection, did not form any lactic acid when kept

TABLE V

O_2 Consumption and Disappearance of Hexosemonophosphate without and with Iodoacetate Poisoning

The muscles were kept anaerobically for 2 hours in phosphate-Ringer's solution containing epinephrine in a concentration of 1:10^7. In some experiments iodoacetate (final concentration 1:10^4) was added during the last half hour. One group of matched muscles was analyzed after the anaerobic period, while the other was shaken in a Warburg apparatus at 20° until the O_2 consumption returned to a steady rate, which was assumed to be the basal O_2 consumption. The muscles were then analyzed for hexosemonophosphate and lactic acid in the same way as their mates. All values are given in mg. per 100 gm. of muscle.

Hexosemonophosphate (as hexose)		Lactic acid		Change in		Carbohydrate equivalent of	
Before	After O_2	Before	After O_2	Hexosemonophosphate	Lactic acid	Total O_2 used	Extra (used
Without iodoacetate (1.7 hrs.)							
80	44	55	48	−36	−7	16	5
112	76	57	55	−36	−2	12	4
89	46	48	38	−43	−10	22	10
87	38	37	34	−49	−3	14	7
Average....				−41	−5	16	7
With iodoacetate (1.8 hrs.)							
112	66	29	21	−46	−8	12	4
111	74	33	33	−37	±0	10	0.6
108	64	27	20	−44	−7	18	1.3
104	65	18	15	−39	−3	17	0.8
Average..........................				−42	−4	14	1.7

anaerobically until rigor developed (average lactic acid content 42 before and 41 mg. per cent after the anaerobic period). The thin muscles of these frogs were removed and those of one side were analyzed at once, while those of the other side were allowed to recover in O_2. It may be seen that hexosemonophosphate disappeared at the same rate as in muscles not poisoned with iodoacetate.[5]

The same result was obtained in experiments in which an accumulation of hexosemonophosphate was produced by keeping thin frog muscles anaerobically for 2 hours in phosphate-Ringer's solutions containing epinephrine and by poisoning them with iodoacetate during the last half hour of the anaerobic period. During the subsequent aerobic period the O_2 consumption was measured in a Warburg apparatus and the disappearance of hexosemonophosphate and lactic acid was determined chemically on the muscles that had been used for the respiration experiments. The results, which are recorded in Table V, show that hexosemonophosphate disappeared aerobically at the same rate whether or not the path to lactic acid was blocked by iodoacetate and that in both cases the total O_2 consumption was insufficient to account for the disappearance of hexosemonophosphate. Hence part of the hexosemonophosphate which disappeared aerobically must have been disposed of in some other way than by oxidation.

It has been shown previously (12) that hexosemonophosphate disappears three times as rapidly under aerobic as under anaerobic conditions. In the latter condition it seems to be converted to lactic acid, while in the former condition, as indicated in the present experiments, it is largely reconverted to glycogen, apparently without being first transformed to lactic acid.

SUMMARY

1. The disappearance of hexosemonophosphate and lactic acid was correlated with the resynthesis of glycogen in frog muscle undergoing aerobic restoration after short tetanic stimulation. More glycogen was resynthesized at 15° than at 20°, while at 25°

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[5] An attempt to demonstrate aerobic resynthesis of glycogen in muscles poisoned with iodoacetate did not give conclusive results owing to marked inequalities in the initial glycogen content of the muscles of the right and left sides.

the glycogen balance became negative. The oxidative quotient for the disappearance of hexosemonophosphate plus lactic acid at 15° was 4.2 but for lactic acid alone it was only 2.4.

2. When the experimental conditions were such that only small amounts of lactic acid could disappear, the amount of glycogen resynthesized during the recovery period exceeded 2.4 times the amount of lactic acid which disappeared, indicating that glycogen was being formed from sources other than lactic acid. The amount of hexosemonophosphate which disappeared was more than sufficient to account for the gain in glycogen from sources other than lactic acid.

3. Hexosemonophosphate disappeared at the same rate in muscle poisoned with iodoacetate as in unpoisoned muscle and the total oxygen consumption was in both cases much less than would have been required for the oxidation of the hexose ester.

4. The experiments indicate that a large part of the hexosemonophosphate which accumulates in muscle during activity is reconverted to glycogen during the recovery period, apparently without being first converted to lactic acid.

BIBLIOGRAPHY

1. Meyerhof, O., *Arch. ges. Physiol.*, **185**, 11 (1920).
2. Meyerhof, O., Lohmann, K., and Meyer, R., *Biochem. Z.*, **157**, 459 (1925).
3. Foster, D. L., and Moyle, D. M., *Biochem. Z.*, **15**, 672 (1921).
4. Hahn, A., Fischbach, E., and Niemer, H., *Z. Biol.*, **92**, 535 (1931–32).
5. Lohmann, K., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 2nd edition, Ergänzungswerk 1, 851 (1933).
6. Fisher, R. E., and Cori, G. T., *Am. J. Physiol.*, **112**, 5 (1935)
7. Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., *J. Biol. Chem.*, **104**, 149 (1934).
8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
9. Wendel, W. B., *J. Biol. Chem.*, **102**, 47 (1933).
10. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **94**, 561 (1931–32).
11. Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, **32**, 968 (1935).
12. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **107**, 5 (1934).

THE APPLICABILITY OF THE BENEDICT-DENIS PROCEDURE TO THE DETERMINATION OF METHIONINE SULFUR*

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A recent paper by Painter and Franke (1) on the validity of the Benedict-Denis (2, 3) method for the determination of sulfur indicates that the method gives sulfur recoveries as low as 40 per cent from methionine. Other studies (4–6) have indicated satisfactory recovery of sulfur from various biological materials, as judged by ability to duplicate the results obtained by sodium peroxide fusion.

Waelsch and Klepetar (7) in describing a micromodification of the Benedict-Denis method report improvement in sulfur recoveries resulting from addition of sodium carbonate to the reagent until a slight alkalinity results. They regard this alkalinity as essential in preventing escape of partially oxidized sulfur (probably SO_2). In the hope that this procedure might result in more satisfactory recoveries of methionine sulfur we have run a large number of Benedict-Denis determinations on constant amounts (0.1000 gm.) of *dl*-methionine, using 10 cc. of the reagent to which were added varying amounts of 4.0 N sodium carbonate solution. By gentle warming with a little water the methionine was brought into solution. The Benedict-Denis reagent and the sodium carbonate were added and the mixture, in a porcelain (sillimanite) dish, was evaporated to dryness on the steam bath and ignited in the usual way, as described by Benedict (2) and by Denis (3). After cooling, the residue was dissolved in excess HCl, the solution was filtered, and sulfate was determined gravimetrically as barium sulfate in the usual way with appropriate blank correction. The dried sample of methionine used gave on analysis 98.00 per cent purity by Kjeldahl determinations and 98.10 per cent by Parr bomb for sulfur. The corresponding sulfur percentage was therefore 21.07 per cent instead of the theoretical 21.50 per cent.

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Table I shows the results obtained from this series. The percentages of sulfur recorded from 0 to 17.5 milli-equivalents of sodium carbonate are the average of from two to six separate determinations between which the maximum variation was about 0.25 per cent. Higher amounts of added alkali produced increasingly erratic results; with 27.5 milli-equivalents of sodium carbonate the sulfur recoveries varied from 9 to 12 per cent (absolute). The effect of added acid is also shown in Table I.

TABLE I

Sulfur Obtained by Benedict-Denis Method on Sample of Methionine

Theory, 21.50 per cent of S, 98 per cent pure by Kjeldahl procedure. 10 cc. of Benedict-Denis reagent were used in each case plus varying amounts of sodium carbonate and of hydrochloric acid.

Na_2CO_3 per 10 ml. reagent	Sulfur, absolute	Recovery of S content
m.-eq.	*per cent*	*per cent*
0	16.66	79.0
2.5	17.68	83.9
5.0	19.04	90.3
7.5	19.46	92.4
10.0	20.16	95.6
12.5	19.88	94.4
15.0	17.75	84.2
17.5	15.76	74.8
20.0	13.90	66.0
22.5	13.23	62.8
25.0	11.86	56.2
27.5	10.52	49.9
HCl per 10 ml. reagent		
5.0	8.92	42.3
10.0	7.98	37.9

These figures demonstrate the beneficial effects of alkali up to amounts of from 10 to 12 milli-equivalents. In this range the sulfur recoveries are approximately 94 per cent of the theoretical

(21.07 per cent) for this sample of methionine. When using 10 cc. of the Benedict-Denis reagent without any added alkali, we still obtain 79 per cent recovery. It is difficult to understand the extremely low recoveries (less than 40 per cent) reported by Painter and Franke for methionine, but lack of information as to the precise conditions employed by these authors makes further comparison impossible. If we are to assume that 50 cc. of the reagent were employed, as it was for determinations on cereals and on proteins, a partial explanation is afforded by some experiments in which we have varied the amount of reagent from 10 to 50 cc. while using a constant amount (0.1000 gm.) of methionine. No sodium carbonate was added. Here we have encountered the unexpected result that the average of 16.66 per cent sulfur obtained with 10 cc. of reagent (see Table I) was lowered to 13.32 and 11.61 per cent by the use, respectively, of 30 and 50 cc. of reagent.

In order to ascertain whether the important factor in the use of sodium carbonate is the pH of the solution or the content of base, we have compared the effect of sodium carbonate with that of an equivalent amount of sodium hydroxide. The effect of this change on the pH of the solution is not great (cupric hydroxide being precipitated instead of basic cupric carbonate) and the final result showed no appreciable difference in sulfur recoveries. Since (see below) the loss of sulfur appears to occur almost exclusively during the ignition rather than the previous evaporation, the actual pH of the solution is probably of less importance.

Comparison of the original Benedict solution (2) with the Denis modification (3) showed that the former produced sulfur yields about 4 per cent less than those with the Benedict-Denis reagent. We have found that the latter yields theoretical results with pure cystine over a wide range of alkalinity.

With methionine, with the Benedict-Denis procedure, no advantage was gained by increasing the time of heating to as much as 2 hours. Under these conditions blank determinations produced as high as 50 mg. of $BaSO_4$ when the ignition was carried out with the unprotected flame of city gas. With gas of high sulfur content, an electric muffle is therefore preferable. The use of platinum dishes is also preferable, as porcelain leads to contamination with silicic acid.

No increase in yield was obtained by following the Benedict-Denis procedure by treatment with perchloric acid. Direct application to methionine of Pirie's perchloric acid method (8) also gave results which were at least 5 per cent too low.

That the low results in the Benedict-Denis procedure are at least partially ascribable to the volatilization of sulfur compounds was proved by determinations in which the evaporation and ignition were conducted in a Pyrex, Kjeldahl flask provided with a receiver, the dried contents of which, analyzed by Parr bomb ignition (9), yielded a substantial proportion of the sulfur lost.[1]

SUMMARY

1. In the determination of sulfur in methionine, the use of the Benedict-Denis reagent produces low results averaging about 79 per cent of theory. Theoretical values were obtained on pure cystine.

2. Addition of varying amounts of sodium carbonate to constant amounts of the Benedict-Denis reagent produces, with methionine, an increase in sulfur recovery, reaching a maximum of about 95 per cent of theory with 10 to 12 milli-equivalents of Na_2CO_3 per 10 cc. of reagent. Cystine recoveries continue to be theoretical.

3. Addition of further amounts of sodium carbonate to 10 cc. of the Benedict-Denis reagent produces a rapid decrease in percentage recoveries, together with very erratic results on methionine. Cystine figures remain theoretical.

4. Addition of acid (HCl) to the determination produces recoveries as low as 38 per cent in the case of methionine. Cystine recoveries remain theoretical.

5. The use of excessive amounts of the Benedict-Denis reagent lowers the recoveries obtained with methionine.

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[1] The well known difficulty of oxidizing sulfones by any method other than sodium peroxide fusion and the possibility of some sulfone formation from methionine indicate that loss could occur in this way. Oxidation of the sulfur before demethylation occurs could conceivably produce a compound of this nature. The inability of the Benedict-Denis procedure to oxidize the sulfur of diphenylsulfone, for example, is shown by the fact that we have obtained from this compound by Parr bomb procedure 14.66 per cent of S (theory, 14.70 per cent), while two Benedict-Denis determinations gave figures of only 1.68 and 1.52 per cent of S.

6. Prolonged ignition is without advantage and is open to the objections that if a gas flame is used the sulfur content of some gases produces unduly high results and that with added alkali too much silicate is removed from the porcelain dish. Heating in an electric muffle is therefore preferable unless a sulfur-free gas is available. The use of platinum ware is also preferable.

7. When low recoveries of methionine sulfur are experienced, the loss of sulfur occurs during the ignition process.

8. Substitution of the original Benedict reagent for that as modified by Denis produces no improvement in sulfur recovery.

BIBLIOGRAPHY

1. Painter, E. P., and Franke, K. W., *J. Biol. Chem.*, **114**, 235 (1936).
2. Benedict, S. R., *J. Biol. Chem.*, **6**, 363 (1909).
3. Denis, W., *J. Biol. Chem.*, **8**, 401 (1910).
4. Halverson, J. P., *J. Am. Chem. Soc.*, **41**, 1494 (1919).
5. Hoffman, W. F., and Gortner, R. A., *J. Am. Chem. Soc.*, **45**, 1033 (1923).
6. Frear, D. E., *J. Biol. Chem.*, **86**, 285 (1930).
7. Waelsch, H., and Klepetar, G., *Z. physiol. Chem.*, **211**, 47 (1932).
8. Pirie, N. W., *Biochem. J.*, **26**, 2041 (1932).
9. Parr Instrument Company, Booklet 113, Moline (1934).

THE ENZYMATIC HYDROLYSIS OF GLUTATHIONE BY RAT KIDNEY

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In previous work it was shown (1, 2) that kidney tissue (rat, rabbit, pig, horse) contains an enzyme which exerts a strong hydrolytic action on reduced and oxidized glutathione. Pancreas also contains the enzyme, although in smaller amounts. Titration data and certain color reactions indicated that hydrolysis proceeded only until all the glycine had been removed, leaving the then unknown γ-glutamylcysteine (3), or γ-diglutamylcystine, in solution.

Further work with various rat kidney preparations has led us to revise our original conclusions. We now find that *both* peptide linkages are completely hydrolyzed, by processes which are without doubt enzymatic. Attempts to isolate γ-glutamylcysteine from kidney-glutathione (GSH) incubation mixtures were not successful. However, a crystalline product, identified as cysteine sulfate, was obtained in 53 per cent yield. Since cysteine is the middle amino acid in the tripeptide molecule, this result indicated either that complete enzymatic breakdown into the constituent amino acids had occurred, or that the glutamylcysteine first formed had been hydrolyzed by chemical action during the attempted isolation. The first alternative proved to be the correct one. Using extremely mild conditions for protein removal (heat coagulation, or removal of an insoluble acetone-ether enzyme preparation by filtration) and then oxygenating the protein-free filtrates, we were able to recover over 70 per cent of the theoretical cysteine as insoluble cystine.

Our original conclusion was based largely on titration data obtained by the method of Linderstrøm-Lang, Weil, and Holter

(4). In this method, titration is carried out in 80 per cent acetone-alcohol mixture, with tetramethylammonium hydroxide as base and thymol blue as indicator. It has now been found that pure glutathione, when titrated to pH 11 to 12 as called for, gives values which are too high by over 0.75 equivalent of alkali. Cysteine, under like conditions, gives the correct value. When proper correction is made for this abnormal behavior of pure glutathione, results are obtained which agree closely with complete hydrolysis of both peptide linkages. The Harris (5) alcohol-formaldehyde-NaOH method, and the Harris (5) HCl method, both of which were found to give correct titrations for pure glutathione and its constituent amino acids, also indicate that both linkages are hydrolyzed.

Oxidized glutathione (GSSG) is likewise readily hydrolyzed into its constituent amino acids by rat kidney, cystine being identified as a product by means of the Sullivan test. Titration data are inconclusive in this case because of interference due to precipitation of cystine during the course of the reaction.

The discovery of the complete enzymatic breakdown of glutathione is of interest in view of the high degree of stability towards enzymes usually attributed to this compound (6–8). Grassmann and coworkers (9) were unable to effect an enzymatic hydrolysis of reduced glutathione, although glycine was removed from the oxidized form by means of pancreatic carboxypolypeptidase. Yet the recent work of Dyer and du Vigneaud (10), who showed that glutathione is able to replace cystine in rats held on a cystine-deficient diet, strongly indicates that hydrolysis to yield cysteine (or cystine) does occur during metabolism.

On the basis of rules formulated by Bergmann *et al.* (11) the splitting of the glycine-cysteine linkage can be satisfactorily accounted for by the action of carboxypolypeptidase. It is more difficult to explain the hydrolysis of the γ-glutamic acid-cysteine linkage. Bergmann and Zervas (12) and Grassmann and Schneider (13) have reported that β-dipeptides of aspartic acid are not attacked by any of the known enzymes, although the α forms are normally hydrolyzed. In view of the close structural similarity between aspartic and glutamic acids, it may be expected that α-dipeptides of the latter will be normally hydrolyzed, as is in fact the case, while γ-dipeptides should be resistant to enzyme attack.

Such γ-dipeptides have not been available for investigation except indirectly in glutathione, in which Grassmann *et al.* (9) were unable to effect hydrolysis. Further work on the nature of the enzyme (or enzymes) responsible for glutathione hydrolysis should show whether we are dealing with a new γ-dipeptidase.

EXPERIMENTAL

Enzyme Preparations—Aqueous kidney extract was made by thoroughly grinding fresh, clean kidneys of albino rats with sand and water (5 to 15 parts, depending on the activity desired), and removing the residual tissue by centrifuging. Acetone-ether preparation was made by mincing the kidneys, extracting four times with 5 volumes of acetone and twice with 5 volumes of ether, drying overnight in air, and sifting the resulting product through a fine sieve. In order to remove as much water-soluble material as possible, 10 gm. of the dried powder were washed four times with 150 cc. of water in a centrifuge tube. The drying with acetone and ether was then repeated.

Isolation of Cysteine Sulfate from Kidney-Glutathione Digests—Attempts to isolate the sulfhydryl compound, resulting from the digestion of glutathione by kidney preparations, by means of copper or lead precipitation methods were not successful. The following mercury procedure was found to be suitable.

1 gm. of GSH was dissolved in 50 cc. of water containing 1 equivalent of NaOH (pH 7). 50 cc. of a 1:5 aqueous rat kidney extract were added and the mixture incubated at 25° for 1 hour. At this time GSH was shown to be absent by the glyoxalase method of Woodward (14). The mixture was deproteinized by addition of 11 cc. of 22 per cent sulfosalicylic acid. The protein-free filtrate was treated with 17 cc. of mercuric sulfate reagent (10 per cent $HgSO_4$ in 5 per cent H_2SO_4) to precipitate the sulfhydryl compound. The mercury precipitate was thoroughly washed at the centrifuge, suspended in 10 cc. of water, and decomposed with H_2S. The HgS was centrifuged down and washed with water, the combined washings and supernatant liquid being gassed with N_2 to remove H_2S. The solution was placed in a vacuum desiccator over H_2SO_4 and evaporated to a syrup which on slight scratching readily set to a crystalline mass. For purification, the product was taken up in 60 cc. of warm 95 per cent alco-

hol, and the solution filtered and concentrated to 10 cc. in a vacuum desiccator over H_2SO_4. Clumps of crystals in rosette formation appeared, which were filtered off, washed with absolute alcohol, and dried. The following analyses identified the product as cysteine sulfate, $(HS—CH_2—CHNH_2—COOH)_2 \cdot H_2SO_4$: found, S 27.8, free H_2SO_4 27.0, N(Kjeldahl) 8.2, cysteine (Sullivan) 71.2; calculated, S 28.2, free H_2SO_4 28.8, N 8.23, cysteine 71.2. The yield obtained in the first crop of the recrystallization was 286 mg., or 53 per cent of the theoretical. The melting point was 175–176° (with decomposition).

For further identification, the same product was prepared from *l*-cystine. 500 mg. of the latter were dissolved in 100 cc. of 2 per cent sulfosalicylic acid, and reduced to cysteine by the addition of zinc dust (total, 0.3 gm.) in small portions during 90 minutes. The reaction was speeded by slight warming, the course of the reduction being followed by iodate titration of the —SH group at 0° (15). After removal of excess zinc by filtration, 16 cc. of $HgSO_4$ reagent were added, and the precipitate carried through the procedure described above. On recrystallization of the crude product from alcohol, a first fraction of 50 mg., melting at 222–224° (with decomposition), was obtained. This contained no H_2SO_4, and was identified as free cysteine by means of the Sullivan reaction and by iodate titration. A sample of free cysteine[1] prepared from the hydrochloride melted at the same point. The second crop of crystals, 220 mg., melted at 176–177° (with decomposition), and has the same microscopic appearance as the cysteine sulfate isolated from the kidney-GSH mixture. Its cysteine content, as shown by Sullivan's test, was identical with that of the cysteine sulfate isolated from kidney-GSH mixtures.

Isolation of Cystine from Kidney-GSH Digests—500 mg. of GSH were dissolved in 75 cc. of water containing 1 equivalent of NaOH. After addition of 2.1 gm. of acetone-ether kidney, the mixture was incubated at 25° in N_2 for 6 hours, at which time the glyoxalase test showed no GSH remaining. The enzyme was removed at the centrifuge and washed with water. The combined washings and supernatant liquid were adjusted to pH 7, oxygenated until the nitroprusside test became negative (90 minutes), and placed in the refrigerator. Overnight, 83 mg. of a white precipitate had formed; on concentration of the liquid, more was deposited, a total of 126 mg. being collected. It was identified as cystine by sulfur analysis (found, 26.7; calculated, 26.9), and by iodate titration after zinc reduction. The yield was 72 per cent of the theoretical.

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[1] Kindly supplied by Dr. G. E. Toennies and Dr. Mary A. Bennett of the Lankenau Hospital Research Institute, Philadelphia.

Amino Acid Titration Data—The irregular behavior of reduced glutathione in the Linderstrøm-Lang, Weil, and Holter (4) titration method is illustrated in Table I. To 1 cc. portions of 0.02 M glycine, glutamic acid, cysteine hydrochloride, and GSH, and 0.01 M GSSG, were added 10 cc. of a 1:1 mixture of acetone and absolute alcohol and several drops of indicator, either thymol blue or phenolphthalein. Titrations were carried out with 0.05 N aqueous NaOH or alcoholic tetramethylammonium hydroxide, to the blue of thymol blue or the pink of phenolphthalein.

TABLE I

Titration of GSH, GSSG, and Amino Acids with Alkali in Acetone-Alcohol

Solution titrated	Indicator	Theoretical titration	Observed titration	
			$(CH_3)_4NOH$	NaOH
		cc.	cc.	cc.
GSH	Thymol blue	0 80	1.14	1 16
"	Phenolphthalein	0 80	0 82	0 80
GSSG	Thymol blue	0 80	0 79	0 82
Cysteine HCl	" "	0 80	0 83	0 83
Glutamic acid	" "	0 80	0 80	
Glycine	" "	0 40	0 42	

Examination of these data shows that pure GSH titrates too high to the extent of over 0.75 equivalent of alkali when thymol blue is the indicator. Since GSSG titrates correctly in the presence of this indicator, it is probable that the high value for GSH is due to partial titration of the —SH group. When phenolphthalein is the indicator, theoretical values are obtained with either base. Cysteine titrates correctly even when thymol blue is the indicator. The same is true for glycine and glutamic acid. In our previous work, with thymol blue as indicator, the —COOH increase during hydrolysis was calculated from the difference between the final titration and that of the original mixture of kidney plus GSH. Since, on the basis of the above evidence, the former value was correct and the latter much too high, the observed —COOH increase was too low, and corresponded more nearly to hydrolysis of one peptide linkage than of two.

Titration of Kidney-GSH Digests—Fig. 1 illustrates a typical GSH-kidney hydrolysis experiment in which the course of the reaction was followed by titration. A mixture was prepared containing 307 mg. of GSH, 50 cc. of water, 1 equivalent of NaOH, and 1.5 gm. of acetone-ether kidney. Incubation was carried out at 25°, with shaking, in an atmosphere of nitrogen. At intervals, 6 cc. samples were centrifuged, and 1 cc. aliquots of the supernatant fluid titrated by the three methods indicated. Blanks were run on a similar mixture of kidney and water under the same conditions. These blanks were small, increasing from 0 at 2 hours to 0.1 cc. of 0.1 N alkali at 24 hours. The hydrolysis was calculated by deducting from the observed titration the kidney

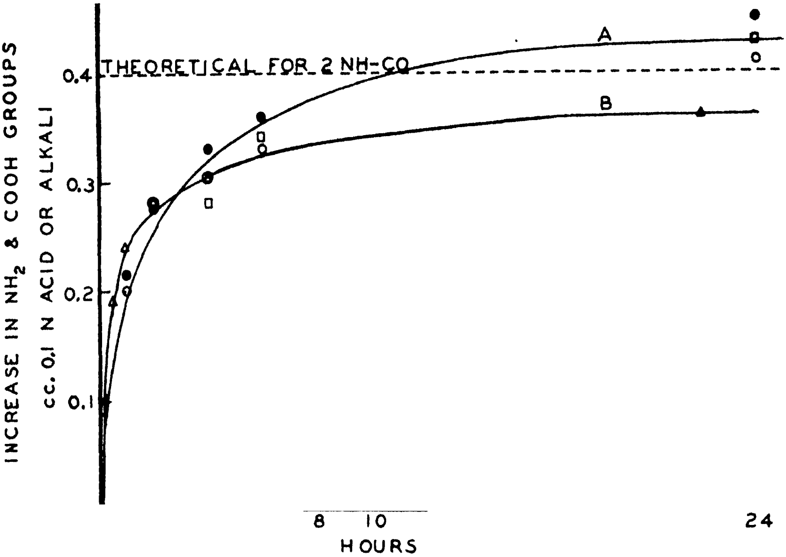

Fig. 1. Hydrolysis of reduced glutathione by rat kidney preparations. Curve A, acetone-ether kidney; Curve B, aqueous kidney extract. ○ and △, Harris NaOH method; □, Harris HCl method; ●, Linderstrøm-Lang, Weil, and Holter method.

blank and the titration of the original GSH. Since, as shown in Table I, GSH titrates high in the Linderstrøm-Lang, Weil, and Holter method, the blank value used for this calculation was not the actually observed high titration, but rather the theoretical for GSH.

All three titration methods used show that complete hydrolysis of both peptide linkages of GSH occurs. The reaction is quite rapid during the 1st hour, the equivalent of one peptide linkage being split; the rate then decreases somewhat, but after 6 hours approximately 90 per cent of all linkages are hydrolyzed. Fig. 1 also shows that fresh kidney extract, like the acetone-ether preparation, hydrolyzes GSH completely. To a solution of GSH neutralized as above was added an equal volume of 1:7.5 fresh, aqueous kidney extract. Incubation was carried out at 25° under anaerobic conditions. 1 cc. samples were titrated at intervals, without further treatment, by the Harris (5) alcohol-formaldehyde method. Blanks were obtained from control kidney extract-water mixtures.

Color Reactions—In confirmation of previous results (2) it was found that Sullivan's (16) test, when applied to such completely digested GSH-kidney reaction mixtures, indicates the presence of free cysteine in nearly theoretical amounts (85 to 95 per cent). The slightly low values are probably due to partial oxidation of the —SH group. γ-Glutamylcysteine,[2] to which the red color development was at first ascribed, gives no color in the test. As reported previously (2), the green color which results when pure glycine is treated with Patton's (17) *o*-phthalaldehyde reagent is not formed in the above reaction mixtures. Instead, one obtains an intense blue color, which we at first also ascribed to γ-glutamylcysteine. However, it has been found that the synthetic dipeptide gives no color with the reagent. Further study has shown that the blue color is obtained when glycine and cysteine are present simultaneously. Cysteine, glutamic acid, or GSH, alone, gives practically no color with the reagent. And the latter two, when present with glycine, do not mask the green color development due to the glycine.

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[2] Acknowledgment is made to Dr. C. R. Harington of the University College Hospital Medical School, London, for supplying a sample of the synthetic product.

Hydrolysis of GSSG by Kidney—307 mg. of GSH were dissolved in 25 cc. of water, neutralized with 1 equivalent of NaOH, and oxygenated until iodate titration showed that practically all of the —SH had disappeared. Two reaction mixtures were prepared, one containing 10 cc. of the GSSG solution and 10 cc. of 1:15 aqueous kidney extract, and the other, 10 cc. of GSSG, 10 cc. of water, and 0.6 gm. of acetone-ether kidney. Both were incubated at 25°, samples being titrated at intervals for —COOH increase by the alcohol-formaldehyde method of Harris.

The titrations increased steadily up to a value, at 4 hours, equivalent to the hydrolysis of approximately half of the total peptide linkages. This value then remained constant up to 24 hours, when the experiment was interrupted. It was at first thought that this indicated a difference from GSH in the behavior of GSSG towards the action of kidney. However, it was observed that at 24 hours the mixture made with fresh extract contained a fairly heavy precipitate. Due to interference of the acetone-ether powder, it was impossible to detect any precipitation in that reaction mixture. It was surmised that the precipitate was cystine, and that its removal from the reaction mixture was the cause of low titrations. This view was shown to be correct by means of the Sullivan cystine (16) test. The reaction mixtures were thoroughly shaken and uniform samples removed. The fresh extract mixture was found to contain 81 per cent of the theoretical amount of free cystine. The acetone-ether mixture was centrifuged and cystine determinations were made separately on the residue and the supernatant liquid. The former contained 40 per cent, and the latter 26 per cent of the theoretically possible free cystine, or a total of 66 per cent. These results, although not as quantitative as those obtained with GSH because of the experimental difficulties involved, nevertheless indicate that all of the peptide linkages of GSSG are attacked by rat kidney preparations.

SUMMARY

Reduced and oxidized glutathione are completely hydrolyzed into their constituent amino acids by an enzyme (or enzymes) present in rat kidney, as indicated by titration data, the Sullivan cysteine test, and the isolation of cysteine (as sulfate) and cystine in high yields from the reaction mixtures. The conclusion drawn

in earlier work, that only glycine is removed enzymatically, must be revised in view of the observation that reduced glutathione titrates abnormally high in the Linderstrøm-Lang, Weil, and Holter tetramethylammonium hydroxide method employed at that time.

BIBLIOGRAPHY

1. Woodward, G. E., Munro, M. P., and Schroeder, E. F., *J. Biol. Chem.*, **109,** 11 (1935).
2. Schroeder, E. F., Munro, M. P., and Weil, L., *J. Biol. Chem.*, **110,** 181 (1935).
3. Harington, C. R., and Mead, T. H., *Biochem. J.*, **29,** 1602 (1935).
4. Linderstrøm-Lang, K., Weil, L., and Holter, H., *Z. physiol. Chem.*, **233,** 174 (1935).
5. Harris, L. J., *J. Biol. Chem.*, **84,** 296 (1929).
6. Hopkins, F. G., *Biochem. J.*, **15,** 286 (1921).
7. Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, **73,** 535 (1927).
8. Kendall, E. C., Mason, H. L., and McKenzie, B. F., *J. Biol. Chem.*, **87,** 55 (1930). Mason, H. L., *J. Biol. Chem.*, **90,** 25 (1931).
9. Grassmann, W., Dyckerhof, H., and Eibeler, H., *Z. physiol. Chem.*, **189,** 112 (1930).
10. Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **115,** 543 (1936).
11. Bergmann, M., Zervas, L., and Schleich, H., *Z. physiol. Chem.*, **224,** 45 (1934).
12. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **224,** 17 (1934).
13. Grassmann, W., and Schneider, F., *Biochem. Z.*, **273,** 452 (1934).
14. Woodward, G. E., *J. Biol. Chem.*, **109,** 1 (1935).
15. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, **97,** 465 (1932).
16. Sullivan, M. X., *Pub. Health Rep.*, *U. S. P. H. S.*, **44,** 1421 (1929).
17. Patton, A. R., *J. Biol. Chem.*, **108,** 267 (1935).

NICOTINIC ACID AS A GROWTH ACCESSORY FOR THE DIPHTHERIA BACILLUS

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In continuation of earlier studies on the nutritional requirements of the diphtheria bacillus (1), the portion of an aqueous tissue extract soluble in 95 per cent alcohol, not extracted by ether from acid solution, but removed by isoamyl alcohol, has been further investigated. It has been found that at least two substances essential for the growth of our test organism can be distilled *in vacuo,* along with a considerable amount of material showing no effect on growth, after both esterification and acetylation of this fraction of the extractives. Addition of relatively small amounts of the lowest boiling and the highest boiling fractions of the distillate, after hydrolysis, to suitable control media, permits abundant growth to take place. The active constituent of the former fraction has been isolated and identified as nicotinic acid and the nature of the material in the higher boiling fraction is still under investigation.

Isolation of Active Crystals

The material used was a hot aqueous extract of liver. After filtration the solution was evaporated *in vacuo* to a thick syrup which was treated repeatedly with 95 per cent alcohol until nothing more was removed. The alcohol extract, again concentrated *in vacuo* to a syrup, was allowed to stand for some time in the cold room and a rather bulky deposit of semicrystalline material was removed and discarded. The solution, strongly acidified with HCl, was extracted three times with isoamyl alcohol, and the extract was concentrated again *in vacuo* with the occasional addition of water to remove the solvent. 1 liter of this material, representing somewhat more than 300 kilos of liver, was made available by the Lederle Laboratories, Inc., Pearl River, New York, in collaboration with Dr. Y. Subbarow of the Department of Biological Chemistry of Harvard Medical School, to whom the writer wishes to express his thanks.

This material was made strongly alkaline to litmus with NaOH and extracted three times with 800 cc. of butyl alcohol, removing considerable inert material. The aqueous residue was neutralized, concentrated *in vacuo* to a very thick syrup, and treated exactly according to a method described by Cherbuliez and Plattner (2) and perfected by Cherbuliez, Plattner, and Ariel (3) for the combined esterification and acetylation of amino acids from protein hydrolysate. No difficulty was experienced at any point, and, by means of an oil pump vacuum of about 0.04 mm., a distillate weighing 94 gm. was obtained. This was divided into a low, middle, and high boiling portion.

The low boiling fraction was carefully redistilled from the Rittenberg (4) apparatus. The procedure was complicated by the presence of considerable quantities of acetamide which crystallized in the side arm, but after two fractionations the bulk of the material showing activity in the growth tests was concentrated into a fraction weighing 0.68 gm. and still containing some acetamide. This fraction was hydrolyzed by refluxing for about 1 hour with 25 cc. of 1.5 N H_2SO_4, an excess of $Ba(OH)_2$ solution was added, the $BaSO_4$ removed, and the solution concentrated *in vacuo* to remove the NH_3 resulting from the decomposition of the acetamide. The excess Ba was then removed accurately with H_2SO_4 and the filtrate concentrated to dryness. The final granular residue was recrystallized from hot ethyl alcohol and the resulting crystals twice more recrystallized from the same solvent. About 10 mg. of crystals were finally obtained.

Identification of Crystals As Nicotinic Acid

During the time that these fractions were being examined, it was brought to the writer's attention, in a personal communication, that Knight (5) had recently found that nicotinic acid was a constituent of a tissue extract which was essential for the growth of the staphylococcus. This substance was, therefore, tested with our organism in various combinations with the acetylated ester distillate fractions. It showed an effect very similar to that of the low boiling ester fraction and of the crystals which had been separated from it. The peculiar inhibition of larger amounts, or rather, the failure of accelerating effect, since growth does not seem to fall below that of controls, was common to both, and this taken together with the physical properties of the crystals suggested their identity.

A mixed melting point determination was carried out in an electrically heated tube so controlled that the temperature was rising about 1° a minute when melting occurred. The following readings were taken.

TABLE I

Comparison of Effects of Crystals Obtained from Liver with Those of Nicotinic Acid

Substance	Bacterial N formed	
	Crystals	Nicotinic acid
mg.	*mg.*	*mg.*
Control	0.34	
0.0001	0.66	0.66
0.00025	0.86	0.86
0.0005	1.07	1.11
0.001	1.51	1.51
0.002	1.54	1.79
0.003	1.89	1.84
0.004	2.15	2.02
0.005	2.20	2.18
0.01	2.20	2.39
0.02	2.04	1.96
0.025	1.62	1.68
0.05	1.54	1.45
0.1	1.37	1.38

	°*C.*
Crystals from liver	234
Same mixed with nicotinic acid	234.4
Nicotinic acid (Eastman)	235

The tubes were heated simultaneously. Slight darkening of the liver preparation and the mixture occurred just before melting, whereas the synthetic material was colorless. The Eastman preparation is stated to melt at 228–229° (the melting point given in the literature). The above readings were uncorrected and made with an uncalibrated thermometer. The conclusion seems warranted that the crystals are nicotinic acid containing a small amount of impurity.

In Table I are shown the results obtained by the addition of increasing quantities of the crystals from liver to one set of control medium and the same quantities of nicotinic acid (Eastman) to a second set. Results are expressed as mg. of bacterial nitrogen obtained on 10 cc. of the medium after 60 hours incubation at 34°, the Allen strain being used. The control medium is constituted as follows, quantities given being for 10 cc.

TABLE II

Effect of Nicotinamide on Growth of Diphtheria Bacillus

Tube No.	Nicotinamide added to control medium	Bacterial N
	mg.	*mg.*
1	None	0.36
2	0.01	0.84
3	0.03	1.34
4	0.05	1.63
5	0.075	2.00
6	0.10	2.24
7	0.20	2.15
8	0.25	1.98
9	0.5	1.88
10	1.0	1.56

Casein-HCl hydrolysate	0.10	gm.
Cystine	0.001	"
Glutamic acid	0.050	"
Lactic acid (as Na salt)	0.10	cc.
Pimelic acid	0.001	mg.
High boiling distillate fraction	1.0	"
NaCl	0.050	gm.
$Na_2HPO_4 \cdot 2H_2O$	0.025	"
KH_2PO_4	0.0035	"
$MgCl_2 \cdot 6H_2O$	0.003	"

The pH is adjusted to 7.4 to 7.6 and the media are autoclaved after tubing at 10 pounds pressure for 10 minutes.

Nicotinamide, the compound of nicotinic acid which may well occur in the liver, since its isolation from heart muscle has recently been described by Kuhn and Vetter (6), was made available for test through the courtesy of Dr. Otto Bessey of the Department of Pathology. Prepared by him from the ester and ammonia, it melted at 122.5° (the melting point given in the literature is 122°). The effect of this substance, when added to the control solution, is shown in Table II.

The effect is quite parallel to that of the free acid except that about 10 times the quantity is required. The opposite evidently was true in the case of the staphylococcus, since Knight reports the amide effective in smaller amounts than the acid.

SUMMARY

1. The method of Cherbuliez and coworkers for distillation of amino acids after both esterification and acetylation has been successfully applied to certain tissue extractives.

2. There seems to be little doubt that the crystalline material, about 10 mg. of which were prepared from the lower boiling portions of a distillate obtained by this method from a concentrate of 300 kilos of liver, is nicotinic acid. The yield naturally is not quantitative, and it is not possible at this stage to calculate back from the concentration at optimal activity to the amount probably present in whole liver. This follows from the observation, not brought out in protocols here given, that the position of the peak of the curve of activity is to some extent dependent upon the concentration of the high boiling fraction. A relationship between the two is suggested which may become clearer as a result of further work which is now in progress.

3. Nicotinic acid exerts its most striking effect on the growth of the diphtheria bacillus, under the conditions employed, in a concentration of about 1.0 microgram per cc. of medium, and is therefore, weight for weight, required in considerably larger quantities than proved to be the case for pimelic acid (7), 0.025 microgram of which per cc. produced its maximal effect.

BIBLIOGRAPHY

1. Mueller, J. H., *J. Bact.*, **29**, 515 (1935). Mueller, J. H., and Kapnick, I., *J. Bact.*, **30**, 525 (1935). Mueller, J. H., and Subbarow, Y., *J. Bact.*, in press (1937).

2. Cherbuliez, E., and Plattner, P., *Helv. chim. acta,* **12,** 317 (1929).
3. Cherbuliez, E., Plattner, P., and Ariel, S., *Helv. chim. acta,* **13,** 1390 (1930).
4. Rittenberg, D., unpublished data.
5. Knight, B. C. J. G., *Nature,* **139,** 628 (1937).
6. Kuhn, R., and Vetter, H., *Ber. chem. Ges.*, **68,** 2374 (1935).
7. Mueller, J. H., *J. Biol. Chem.*, **119,** 121 (1937); *J. Bact.*, in press (1937).

THE DETERMINATION OF ESTRIN IN URINE WITH THE PHOTOELECTRIC COLORIMETER

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Kober (1) in 1931 discovered that when estrin is heated with concentrated sulfuric acid and phenol it gives rise to a yellow color. On addition of water and reheating the mixture, this color is converted into a highly specific pink color. Unfortunately the pink color is unstable, and in addition the reagent reacts with other substances present in urine extracts to produce a brown color which masks the pink due to the estrin. In 1934 Cohen and Marrian (2) tried to overcome these difficulties by modifying the method for use with the Lovibond tintometer. They plotted color development curves for each sample in an effort to measure the unstable pink color at its maximum intensity. They also tried to correct for the interfering effect of the brown color by measuring the number of red Lovibond units in the sample before and after the pink color is discharged with hydrogen peroxide. This method of correction was only approximate, however, because hydrogen peroxide also discharges a considerable amount of the brown color.

In a preliminary report in 1936 (3) we described a method for stabilizing the pink color, and a method of measuring it in the presence of the brown contaminant by means of the photoelectric colorimeter (4). An attempt has also been made by Pincus, Wheeler, Young, and Zahl (5) to carry out the analysis on the Pulfrich photometer, but this method is applicable only to extracts of late pregnancy urine, since no correction was made for the brown color, the interfering effect of which naturally becomes

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more and more important as the concentration of estrin in the urine is diminished.

Spectrophotometric Studies[1] *and Selection of Color Filters*[2]—The spectrophotometric curve of the pink color obtained from 30 micrograms of estriol (m. p. 278°) is shown in Curve 1 of Fig. 1, in which percentage light transmission is plotted against wave-length. The pink colors obtained from estrone and estradiol are not shown on the figure because the curves are qualitatively identical with that of estriol, although there are slight differences between the amounts of pink color obtained from equal amounts of the three compounds. Comparison of Curve 1 with Curve 2 (which corresponds to 60 micrograms of estriol) illustrates the proportionality between the amount of estriol and the depth of the characteristic absorption band at 522 $m\mu$, and shows how the measurement of the light absorption at this wave-length may be used to determine the amount of estriol present. This measurement may be made accurately and conveniently by means of a photoelectric colorimeter equipped with a filter (Curve 5 of Fig. 1) which transmits a narrow spectral band in the vicinity of 522 $m\mu$. Measurements made with a single such filter will, however, be grossly inaccurate when applied to the colors from urine extracts, because the brown color due to non-estrin substances (Curve 3 of Fig. 1) also absorbs light at this wave-length. A comparison of Curves 1 and 3, however, shows that the brown color absorbs even more strongly at 420 $m\mu$, while the pink color is almost completely transparent at this wave-length. This is also well shown by Curve 4 which corresponds to the colored mixture obtained when 30 micrograms of estriol are added to the estrin-free extract from which Curve 3 was made. It is evident that the light absorption of this mixture at 420 *mμ* is due to the brown component alone, while that at 522 *mμ* is the sum of effects due to both brown and pink components. If, therefore, we measure the light absorption of each mixture not only at 522 *mμ* but also at 420 *mμ* (using the filter shown in Curve 6 of Fig. 1), we can calculate the light absorption of the brown component at 522 *mμ* from its measured absorption at 420 *mμ*, and hence by subtraction we can obtain the absorption at 522 *mμ* due to the pink component only. This is the principle employed in our method, but in practise it is not necessary to make a separate calculation for each sample, because a simple nomogram[3] (Fig. 2) can be constructed on which the readings with the two filters (522 and 420 *mμ*) may

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[1] The great difference between these curves and those reported by Pincus, Wheeler, Young, and Zahl (5) is explained by the fact that these authors have used the procedure of Cohen and Marrian and therefore did not measure the pure pink color, but a mixture of pink and unconverted yellow. The differences reported by Pincus *et al.* (5) between the curves for estriol, estrone, and estradiol are due merely to slight differences in the extent to which the yellow color from these three compounds is converted into pink by the method used by these authors.

[2] Exact duplicates of the filters used in this technique can be obtained from the Rubicon Company, 29 North Sixth Street, Philadelphia. The complete photoelectric colorimeter is also obtainable from the Rubicon Company.

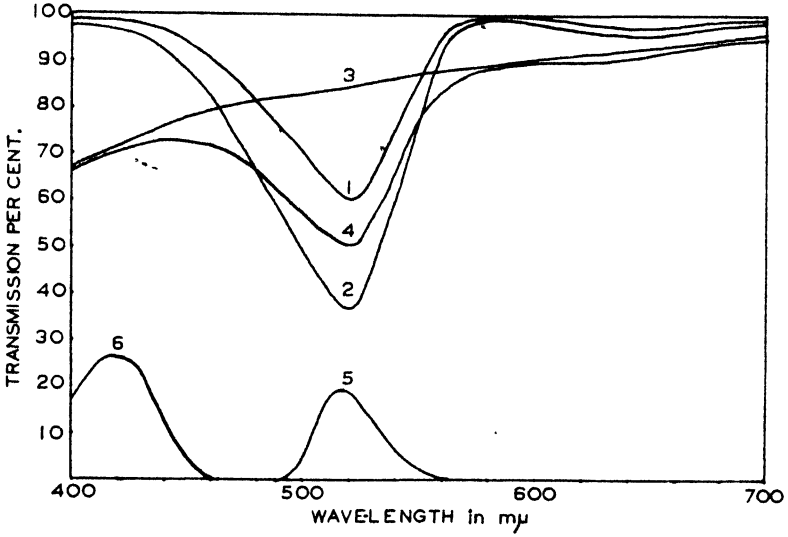

FIG. 1. Spectrophotometric curves of (Curve 1) pink color from 30 micrograms of estriol, (Curve 2) pink color from 60 micrograms of estriol, (Curve 3) brown color from 1 cc. of an estrin-free urine, (Curve 4) mixed color from 1 cc. of the same extract plus 30 micrograms of estriol, (Curve 5) green filter used in measurement of absorption at 522 *mμ*, and (Curve 6) blue filter used in measurement of absorption at 420 *mμ*.

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[3] Since all photoelectric colorimeters of the type used by us are interchangeable with respect to calibration, the same nomogram can be used universally. We have therefore prepared a number of photostats of the chart which we will be glad to furnish on request.

be used as coordinates to specify a point on the chart from which the amount of estrin in the solution can be read directly. The

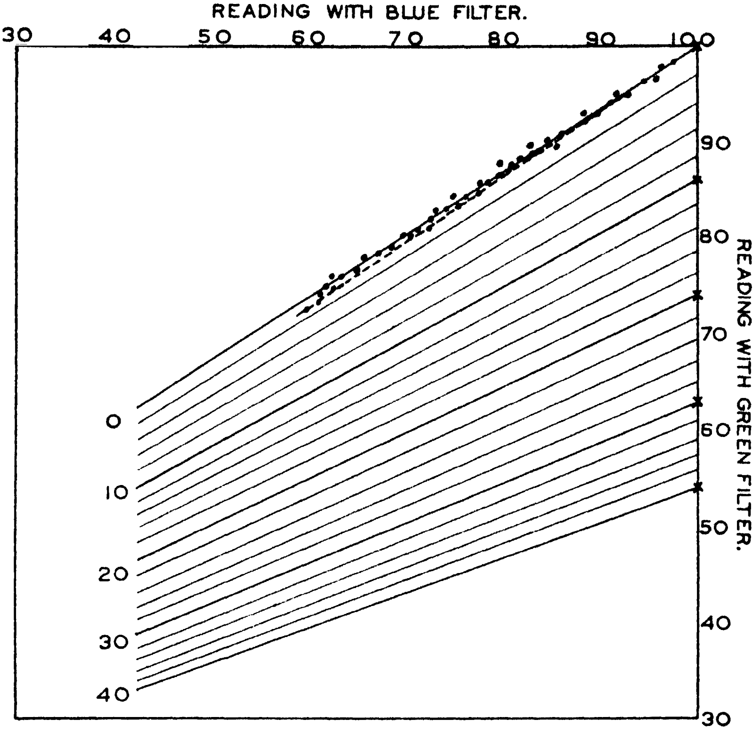

Fig. 2. Calibration chart. The figures attached to the lines represent micrograms of estriol. The slope of the line[5] labelled 0 was determined from measurements on 50 estrin-free urinary extracts, and thus corresponds to the "average brown color." All pure pink colors (obtained from pure estriol) will fall on the right-hand axis of coordinates as shown by the points marked X, which correspond to 10, 20, 30, and 40 micrograms of estriol respectively. The position of any intermediate line between the pure brown and the pure pink is readily obtained by multiplying the ordinate of any point on the brown line by the ordinate of the amount of estrin to which the line corresponds.

Results obtained from the chart are only accurate if the galvanometer reading with the blue filter is above 60, since the extrapolation of the 0 estrin line below this point is not strictly accurate.

construction of the nomogram is described in the legend to Fig. 2.

It is obvious that the above method of correcting for the interfering effect of the brown color is only valid if the spectrophotometric characteristics of the brown color are sufficiently reproducible to allow us to deduce the light absorption at 522 mμ from the measured absorption at 420 mμ. A careful spectrophotometric study of the brown colors developed from a wide variety of urinary extracts has convinced us that this hypothesis is essentially correct, and has led us to believe that the brown color is largely produced by a non-specific charring of organic material in the extracts. Small variations in the quality of the color do however occur, and we shall show below that the magnitude of these variations is the most important limiting factor in the accuracy obtainable by this method.

With regard to the specificity of the reaction, we may state that we have found no other substance likely to be present in human urine which produces a pink color with the reagent used.

Discussion of Factors Affecting the Kober Reaction—The factors affecting the intensity and stability of the pink color are (*a*) the composition of the reagent and the amount used, (*b*) the length of time during which the estrin is heated with the reagent to develop the intermediate yellow color, (*c*) the amount of water added to the yellow mixture, and (*d*) the time of reheating required to convert the yellow color into the specific pink end-product. After a careful study of all these factors we have found that 3 cc. of the reagent[4] recommended by Cohen and Marrian

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[4] The effect of varying the ratio between phenol and sulfuric acid in the reagent was studied, and it was found that the reagent finally adopted by Cohen and Marrian (2) was quite satisfactory (3.6 parts of phenol mixed with 5.6 parts of sulfuric acid). In order to obtain reproducible results the following instructions should be carefully followed: The phenol must be freshly redistilled and only pure sulfuric acid should be used (Merck's reagent quality has been found to be satisfactory). The sulfuric acid should be added slowly to the phenol, care being taken to exclude all traces of moisture and to prevent the temperature of the mixture from rising. The reagent should be kept in a glass-stoppered flask to exclude moisture and dust particles. A standard solution of pure estriol (1 cc. = 30 micrograms) should be used to check the efficiency of each new lot of reagent before use. The reagent should be checked every week. The nature of the reagent makes accurate measurement with a pipette difficult, and precludes the use of a greased stop-cock. We have used a tapless 25 cc. burette filled from below by suction, the flow being regulated by a pinchcock on a rubber tube attached to the upper end.

(2) are sufficient to develop the maximum pink color from at least 100 micrograms of estriol, this being the maximum amount used in our technique. Under the conditions of our method it was found that in order to obtain maximum color, a period of initial heating of 20 minutes with the reagent was necessary before the addition of water. The stability and intensity of the pink color is dependent to a great extent upon the amount of water added and the time of reheating. By varying these two factors different equilibrium states are reached. However, it was observed that a complete conversion of yellow to pink was obtained if exactly 3 cc. of water were added and the mixture reheated for 3 minutes. The pink color so developed, after cooling and addition of 10 per cent sulfuric acid, is quite stable. The colors obtained by this procedure differ from those of Pincus, Wheeler, Young, and Zahl (5) (with the Cohen-Marrian technique (2)) in that complete conversion of yellow into pink is secured by increasing the time of preliminary heating.

Preparation of Urinary Extracts

The greater part of the estrin in urine occurs in a combined form. Although estriol glucuronide in the pure state gives a satisfactory color with the reagent without previous hydrolysis, the composition of urine or urinary extracts is such that it is impossible to carry out the color reaction directly. The conversion of the combined estrin to the free form by means of hydrolysis with subsequent extraction with ether constitutes at present the only reliable method by which a large part of these impurities may be removed without too great a loss of estrin.

We have modified the method of Cohen and Marrian (6) by applying the hydrolysis to a butyl alcohol extract of urine rather than to the urine itself. By this procedure both free and combined types of estrin are extracted satisfactorily. The amount of estrin remaining in the urine after such an extraction is negligible as assayed on the immature rat.

The use of butyl alcohol extracts has several advantages.. The control of the acidity during the process of autoclaving is facilitated, and the time of heating necessary for complete hydrolysis of the combined estrin is shortened. There is also less brown color developed in the final extract.

Extract of urines from many different sources were investigated and the amount of acid and the time of heating were varied in order to ascertain the optimum conditions for hydrolysis of the conjugated estrogens. Phosphoric, tartaric, and hydrochloric acids were used. Hydrochloric was the only acid that gave satisfactory recoveries. The extracts are first made acid to Congo red paper, and it has been found that an additional amount of at least 0.5 cc. to 0.6 cc. of concentrated hydrochloric acid per 100 cc. of original urine was necessary to hydrolyze completely the conjugated estrogens. If more than 1 cc. of acid was added, there was a slight destruction of the estrin. Heating for 1 hour in the autoclave at 120° was not sufficient; 2, 3, and 4 hours gave a maximum recovery of estrin. The concentration of the extracts during autoclaving plays an important rôle in the efficiency of the hydrolysis and 1 cc. of the extract should be equivalent to 2 cc. of original urine.

We have tried the various methods of purification recommended by Cohen and Marrian (2) such as washing the ether with aqueous sodium carbonate or bicarbonate (to remove acidic substances), and extracting with 0.1 N and N sodium hydroxide, reacidifying, and extracting with toluene (to remove inhibiting substances such as pregnanediol and cholesterol). Almost colorless extracts can be obtained by such purification, but the loss of estrin in the process varies between 20 and 60 per cent. No advantage is therefore gained by further purification, since any increase in color due to the removal of inhibiting substances is more than annulled by the resulting loss of estrin.

Method

Extraction of Urine—200 to 300 cc. of urine are acidified (to Congo red paper) with hydrochloric acid and are extracted in a separatory funnel four times with butyl alcohol, 20 to 30 cc. of the solvent being used for each extraction. The butyl alcohol fractions are united and washed once with 5 cc. of water. Occasionally the urine forms an emulsion with butyl alcohol; this may be rapidly broken up by centrifuging the mixture. The butyl alcohol is evaporated to dryness under reduced pressure, the residue is dissolved in 2 cc. of 95 per cent ethyl alcohol, and enough water is added to make the solution 10 per cent ethyl alcohol.

Hydrolysis of Extracts—The extract is diluted so that 1 cc. equals 2 cc. of original urine. It is heated on a hot-plate, the alcohol is boiled off, and the mixture is acidified to Congo red paper with concentrated hydrochloric acid. An additional 0.6 cc. of acid for each 100 cc. of urine is added; then the flask is covered with tinfoil and autoclaved at 120° for 3 hours.

Ether Extraction of Autoclaved Extracts—After hydrolysis the mixture is extracted four times with freshly distilled ether or with ether washed with a 1 per cent solution of ferrous sulfate (this process breaks up any peroxides present in the ether). The ether extracts are combined and washed twice with small amounts of water, evaporated almost to dryness, and the residue is dissolved in hot alcohol. 1 cc. of the alcohol extract should be equivalent to about 5 cc. of the original urine, if the expected estrin content is below 1000 micrograms per liter. In late pregnancy urine an attempt should be made to prepare a final extract in which 1 cc. contains from 5 to 30 micrograms of estrin. It may be necessary to centrifuge the alcohol extract to remove small particles of precipitate which would be charred by the reagent. The colorimetric determination is carried out on an aliquot of this extract containing 5 to 30 micrograms of estrin.

Colorimetric Determination—1 or 2 cc. of the urinary extract (or other solution containing estrin) are evaporated to dryness in a colorimeter test-tube. Evaporation may be carried out in a boiling water bath by drawing a current of air filtered through glass wool through each tube, after which the tubes are placed in a desiccator over sulfuric acid for 1 hour. 3 cc. of a reagent[4] consisting of a mixture of 3.6 parts of phenol and 5.6 parts of concentrated sulfuric acid are run in from a burette, and the tube is placed in a boiling water bath for 20 minutes. Care must be taken to exclude moisture and particles of dust which might be charred by the reagent (rubber stoppers wrapped in lead-foil are useful). The tube should be removed from the bath twice during the first 10 minutes and shaken to insure complete mixing of the estrin with the very viscous reagent. At the end of 20 minutes, the tube is transferred directly to an ice and water bath for at least 5 minutes. 3 cc. of water are added and the contents thoroughly mixed with a stirring rod which is left in the tube throughout the remainder of the process. During the addition of water and mixing of the contents the tube is not removed from the ice bath, as it is essential that the temperature should not rise at this stage. The tube is then replaced in the boiling water bath for 3 minutes, cooled again for 5 minutes in the ice bath, and finally 9 cc. of 10 per cent sulfuric acid are added to bring the total volume to 15 cc. The contents are mixed; the tube is thoroughly dried on the outside and read in the photoelectric colorimeter first with the blue and then with the green filter, and the amount of estrin present read from the calibration chart.

With each set of tubes, a blank tube containing no estrin is carried through the entire procedure and all readings are made after the initial galvanometer deflection is set to 100 with this blank tube in place. The readings should be made as quickly as possible, although no fading occurs in 60 minutes even if the temperature rises as high as 20°. Duplicate determinations should be made on each extract. If too concentrated an extract is used, the galvanometer reading may be outside the linear portion[5] of the calibration (which is limited to readings between 30 and 100 with the green filter and 60 and 100 with the blue filter), and it is then advisable to repeat the entire determination with a suitably diluted extract. With a large water bath with a built in test-tube rack, a single operator may conveniently handle twenty tubes at a time. The various heating times can be kept accurate by inserting and removing the tubes in a definite order.

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[5] It is clear that if measurements made with the two filters on the brown component obeyed theoretical spectrophotometric relations, the line representing the brown color would not be perfectly straight, since it would correspond to a linear relation between log (reading with blue filter) and log (reading with green filter). There are however two considerations which justify the approximation involved in the use of a straight line to represent this relation. In the first place the width of the spectral bands transmitted by the filters is sufficient to account for a slight variation from theoretical predictions. Moreover the magnitude of the deviation from the theoretical line (shown dotted in Fig. 2) throughout the portion of the chart on which the readings are usually made is so small compared with the known uncertainty zone due to variations in the character of the brown color, that the error may justifiably be neglected in face of the resulting simplification of the construction of the chart.

Determination of Pure Estrin

If the technique is to be used for measuring estrin in pure solution, readings need theoretically be made with the green filter only, since no brown contaminant will be present. It is however difficult to be certain that any particular preparation contains no impurity and we have therefore treated all samples as potentially impure, always making readings with both filters, and obtaining the result from the same nomogram used for measurements on urinary extracts.

Results

In determinations on pure estrin the method can be used with as little as 1 microgram and an accuracy of ±3 per cent can be obtained in measurements on as little as 10 micrograms. We have made calibration curves for preparations of estriol (m. p. 278°), estradiol (m. p. 168°), and estrone (m. p. 252°). We have also tested the international standard estrone, and have found that it gives the same amount of color as our preparation. The amounts of color produced from equal amounts of estriol and estrone are in the ratio of about 1.0:1.1, which corresponds fairly well with the inverse ratio of the molecular weights which is 1.0:1.07. This supports the view, already made probable by the similarity of the spectrophotometric curves, that the colored compounds obtained from estriol and estrone are identical. We have also tested two preparations of the sodium salt of estriol glucuronide kindly supplied us by Dr. G. F. Marrian. These samples contained respectively 0.5 mole of methyl alcohol and 0.5 mole of water of crystallization, the calculated percentages of estriol being 57.5 and 58.2 per cent. Both samples gave the usual pink color without previous hydrolysis, the measured estriol content being 50 per cent in each case.

In determinations on urinary extracts the accuracy obtainable by the method is limited by the extent to which the spectrophotometric characteristics of the brown colors developed from successive extracts differ from the arbitrarily selected "average brown color" on which our calibration curve is based. The magnitude and distribution of these variations are graphically recorded by the scatter of the individual points in Fig. 2 about the line taken to represent the average brown color. It is clear that the maximum deviation from the average would never account for an error of more than ±2 micrograms of estrin, but the fact that an error of this magnitude *may* occur in any given sample means that the maximum obtainable accuracy in any determination on urinary extracts is ±2 micrograms. Since with our present method of purification a determination can seldom be carried out on an amount of extract corresponding to more than 10 cc. of urine, this means that each determination is subject to an error of ±200 micrograms per liter of urine. The method is therefore not applicable to urines containing much less than 500 micrograms per liter, although an accuracy of about ±5 per cent can be obtained when the estrin content of the urine rises to 5000 micrograms per liter. The method may therefore be used with safety any time after the 3rd month of pregnancy, and becomes quite accurate by the 4th month.

Table I contains a few of the results of a number of typical experiments which were carried out to test the efficiency of various steps in the method as well as the over-all accuracy when applied to urinary extracts. From the results shown in Table I, the following conclusions are drawn:

1. Estriol added to extracts of non-pregnancy urine is recovered with a mean error of not more than 10 per cent. This recovery is not appreciably improved when the extracts are purified beyond the ether stage. Estriol added to extracts of pregnancy urine is recovered with about the same accuracy. This indicates that inhibiting substances are seldom present in significant amounts in extracts prepared by our method.

2. The loss of estriol added to both pregnancy and non-pregnancy urine and subsequently carried through the entire procedure of extraction, hydrolysis, and color development depends somewhat on the concentration of estriol in the urine, being greater at low concentrations. In concentrations such as are encountered in determinations on pregnancy urines after the 4th month, the loss in the entire procedure can always be kept below 10 per cent.

3. The recovery of sodium estriol glucuronidate under similar conditions is at least as good as that obtained with estriol.

4. There is a considerable amount of destruction when pure estriol and sodium estriol glucuronidate are hydrolyzed in aqueous solution without the protective presence of urine extract.

5. The inhibiting effect of pregnanediol when added to estriol or urinary extracts after hydrolysis is fairly high, but so much of

TABLE I

Recovery of Estrin

	Estrin in sample	Estriol added	Amount observed	Recovery
	micrograms	*micrograms*	*micrograms*	*per cent*
Recovery of estriol added to final extract	0	20	19	95
	0	40	39	97.5
	4.3	21.9	25.5	97.5
	10	21.9	31.5	98.8
Recovery of estriol added to 200 cc. urine	0	90	80	89
	0	2000	1900	95
	82	100	164	90
	400	500	850	94
		Sodium estriol glucuronidate added		
Recovery of estriol glucuronidate added to urine	0	300	275	92
	0	500	475	95
	10	90	90	90
	88	400	475	97
	Amount used			
Recovery of pure estriol and pure estriol glucuronidate from aqueous solution	150		96	64
	30		16.5	55
	25 (Glucuronidate)		12	48
	15 "		7	46.5
	Estrin in urine	Sodium pregnanediol glucuronidate added to urine		
Inhibition of color by pregnanediol	62.5	0	62.5	100
	62.5	150	57.5	92
	62.5	300	50.5	81

the pregnanediol is destroyed or removed during the hydrolysis and subsequent purification that the inhibiting effect of pregnanediol in the amounts in which it occurs in pregnancy urine never exceeds 10 per cent.

SUMMARY

1. The colors produced by the Kober reaction for estrin in urine have been studied spectrophotometrically in order to select color filters for use with the photoelectric colorimeter in the determination of estrin in urine.
2. The factors affecting the intensity and stability of the pink color have been studied, and the optimum conditions determined for the development of stable colors from pure estrin and from estrin in urinary extracts.
3. The preparation of urinary extracts has been studied in order to determine the conditions under which the maximum yield of estrin is obtained.
4. A technique based on the above investigations has been developed for the determination of estrin in pregnancy urine.
5. Difficulties in the purification of urinary extracts prevent the application of the method to urines containing less than 200 micrograms per liter, but the accuracy of the determination increases as the concentration of estrin in the urine rises, and reaches a maximum of ±5 per cent at 5000 micrograms per liter.
6. In determinations on pure estrin quantities as small as 1.0 microgram can be detected, and amounts of the order of 10 micrograms can be measured with an accuracy of ±3 per cent.

BIBLIOGRAPHY

1. Kober, S., *Biochem. Z.*, **239,** 209 (1931).
2. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, **28,** 1603 (1934).
3. Venning, E., Evelyn, K. A., and Harkness, E. V., *Tr. Roy. Soc. Canada* (1936).
4. Evelyn, K. A., *J. Biol. Chem.*, **115,** 63 (1936).
5. Pincus, G., Wheeler, G., Young, G., and Zahl, P. A., *J. Biol. Chem.*, **116,** 253 (1936).
6. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, **29,** 1577 (1935).

THE ABSORPTION SPECTRA OF COMPOUNDS RELATED TO THE STEROLS*

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The bile acids, the sex hormones, antirachitic agents, carcinogenic hydrocarbons, certain heart poisons, some alkaloids, and many other sterol-like substances, with their varied and important physiological properties, have become the focus of attention for many investigators within the past few years. The identification and determination of the concentration of these substances and their derivatives will rest upon accurate spectrographic data, since other physical properties, *e.g.*, melting points, specific rotations, and refractive indices, are in most cases not sufficiently characteristic. While the absorption spectra of many such compounds have been reported, little attempt has been made to determine absolute absorption coefficients with high precision.

In the course of our work we have made accurate studies of the spectra of some compounds related to the sterols, several of which are reported here. These were all obtained directly by the use of a monochromator and photocell. A complete discussion of the method and its accuracy has been presented in an earlier paper (1). In all cases, pure samples of the substances studied were obtained, and for such samples the absorption spectra are not in error by more than 1 or 2 per cent.

EXPERIMENTAL

The compounds studied were considered pure when the absorption spectra of the products from two successive crystallizations or distillations were identical within the limits of error of the method employed. All solid preparations were dried in a vacuum (10^{-5}

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TABLE I

Absorption Data for Phenanthrene in Ethyl Alcohol

Sample weight, 16 to 200 mg.

	Molecular α*	Maximum deviation, 3 runs	Concentration	Cell thickness	Slits	Spectral range isolated†
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2430 ± 5	49,000	1000	0.00090	1.0	0.10	6.6
2500	64,500	700	0.00090	1.0	0.08	6.1
2730	13,200	200	0.0040	1.0	0.08	8.5
2800	10,450	100	0.0050	1.0	0.08	9.5
2920	13,000	150	0.0040	1.0	0.08	11.2
3080	186	2	0.290	1.0	0.055	9.4
3140	222	4	0.290	1.0	0.055	10.2
3220	244	4	0.410	0.496	0.055	11.3
3285	282	2	0.410	0.496	0.055	12.1
3365	224	3	0.410	0.496	0.04	9.6
3445	253	5	0.410	0.496	0.03	7.8
Minima						
2250 ± 5	8,610	200	0.0053	1.0	0.20	9.6
2440	47,600	500	0.0090	1.0	0.10	6.8
2690	11,600	200	0.0040	1.0	0.08	4.0
2775	9,430	100	0.0050	1.0	0.08	4.3
2865	5,200	100	0.0050	2.0	0.05	6.5
3065	172	3	0.290	1.0	0.055	9.2
3100	167	3	0.290	1.0	0.055	9.7
3180	153	2	0.290	1.0	0.055	10.8
3255	162	4	0.410	0.496	0.055	11.8
3330	119	2	0.410	0.496	0.055	12.6
3410	65.7	0.6	0.410	2.0	0.03	7.5

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* α is defined by the equation $I_0/I = 10^{\alpha c l}$ where c is expressed in moles per liter and l in cm.

† "Spectral range isolated" refers to the wave-length limits of the radiation passing through the second slit of the monochromator. This region is not completely isolated from the continuous spectrum. It may be shown that, for any monochromator with widths of Slit 2 and image of Slit 1 of the monochromator equal to each other, only one wave-length will be completely transmitted by Slit 2 at any single drum setting. The intensities of other wave-lengths transmitted by Slit 2 decrease linearly and become 0 at the two extremes of the region isolated. Experimentalists often state the dispersion, with no mention of the slit width employed, and lack of these data prohibits strict comparison of their spectra with others obtained by the same general method. The spectral region isolated must often be the same in two cases before differences in measuring systems and sensitivities may be dismissed and before other problems of chemical purity, source, constitution, etc., of the samples can be considered. The effect of slit width upon the spectral region isolated and upon the magnitude of absorption coefficients in the neighborhood of maxima and minima is considered in more detail in a paper by Hogness, Zscheile, and Sidwell (1).

mm. of Hg) at room temperature. The compounds were prepared for use as described below.

Phenanthrene—Commercial phenanthrene was purified by the method of Cohen and Cormier (2) and recrystallized three times from ethyl alcohol. The dried product melted at 99.6°. All absorption bands of anthracene were absent in the spectrum of this preparation. The absorption data for this compound appear in Table I.

Phenanthrenequinone—A portion of the purified phenanthrene was oxidized to phenanthrenequinone by chromic acid in glacial acetic acid solution. The product was recrystallized once from glacial acetic acid and three times from absolute ethyl alcohol. The melting point of the purified product was 206.8°. Absorption data appear in Table II.

TABLE II

Absorption Data for Phenanthrenequinone in Ethyl Alcohol

Sample weight, 17 to 27 mg

	Molecular α	Maximum deviation, 2 runs	Concentration	Cell thickness	Slits	Spectral range isolated
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2570 ± 10	29,400	300	0.00210	1.0	0.06	5.1
2650 10	31,400	300	0.00210	1.0	0.06	5.7
3225 20	4,550	50	0.0078	2.0	0.04	8.3
4125 20	1,570	20	0.0192	2.0	0.02	2.3
Minima						
2275 ± 5	7,000	100	0.0192	0.496	0.10	5.0
2600 5	27,700	200	0.00210	1.0	0.06	5.3
2925 20	2,000	70	0.00480	7.0	0.04	5.7
3800 20	1,010	30	0.003	7.0	0.03	10.5

Ergosterol—The product of Mead Johnson and Company was crystallized first from ethyl alcohol, then from benzene, and finally twice from isooctane. Absorption data appear in Table III.

7-Dehydrocholesterol—The sample was prepared by the method of Windaus, Lettre, and Schenck (3). The product was first separated from ether and then recrystallized from purified absolute ethyl alcohol. The melting point of the product was 126°. Absorption data appear in Table IV.

Theelin—The product of Schering-Kahlbaum was recrystallized three times from purified absolute ethyl alcohol. A product from Dr. E. A. Doisy produced the same absorption spectrum.

TABLE III

Absorption Data for Ergosterol

	Molecular α	Maximum deviation, 3 runs	Concentration	Cell thickness	Slits	Spectral range isolated
Isooctane solution; sample weight, 45 mg.						
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2520 ± 10	3,800	30	0.055	0.496	0.06	4.7
2620 5	6,750	60	0.026	1.0	0.07	6.3
2710 5	9,700	70	0.013	1.0	0.07	7.3
2810 5	10,050	70	0.013	1.0	0.07	8.4
2930 10	5,800	50	0.018	1.0	0.06	8.6
3225 15	39	3	0.440	7.0	0.04	8.3
3380 20	28	3	0.440	7.0	0.03	7.3
Minima						
2295 ± 5	1,450	50	0.080	1.0	0.12	6.3
2630 5	6,590	60	0.018	1.0	0.07	6.5
2750 5	7,830	70	0.013	1.0	0.07	7.7
2880 5	5,080	20	0.018	1.0	0.06	8.0
3175 15	37	3	0.440	7.0	0.04	7.8
Ethyl alcohol solution; sample weight, 45 mg.						
Maxima						
2520 ± 10	3,860	100	0.0230	1.0	0.06	4.7
2620 5	6,940	40	0.0170	1.0	0.06	5.4
2710 5	10,000	100	0.0120	1.0	0.05	5.2
2820 5	10,600	100	0.0120	1.0	0.05	6.1
2930 5	6,060	50	0.0120	2.0	0.05	7.1
3250 15	37	2	0.430	7.0	0.06	13.0
3375 20	26	2	0.870	7.0	0.04	9.7
Minima						
2300 ± ?	1,430	10	0.160	0.496	0.10	5.2
2630 5	6,850	50	0.0190	1.0	0.05	4.4
2755 5	8,580	100	0.0120	1.0	0.05	5.5
2890 5	5,450	100	0.0120	2.0	0.05	6.7
3175 15	35	2	0.430	7.0	0.06	11.7

Androsterone—A pure sample was kindly supplied by Professor L. Ruzicka. Absorption data for theelin and androsterone appear in Table V.

TABLE IV

Absorption Data for 7-Dehydrocholesterol in Ethyl Alcohol

Sample weight, 10 mg

	Molecular α	Maximum deviation, 2 runs	Concentration	Cell thickness	Slits	Spectral range isolated
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2520 ± 10	4,110	150	0 0290	1 0	0 10	7 8
2625 5	7,400	100	0 0190	1 0	0 09	8 3
2710 5	10,400	100	0 0120	1 0	0 09	9 4
2815 5	10,750	100	0 0120	1 0	0 09	11 0
2930 5	6,150	150	0 0190	1 0	0 08	11 4
3210 15	36		0 430	7 0	0 08	16 1
3360 20	25		0 732	7 0	0 05	11 9
Minima						
2300 ± 15	1,500	50	0 160	0 496	0 16	8 3
2760 5	8,830	100	0 0120	1 0	0 08	8 8
2880 5	5,530	100	0 0190	1 0	0 08	10 6

TABLE V

Absorption Data for Theelin and Androsterone in Ethyl Alcohol

	Molecular α	Maximum deviation, 3 runs	Concentration	Cell thickness	Slits	Spectral range isolated
Theelin; sample weight, 7 2 mg						
Å.			*gm per l.*	*cm.*	*mm.*	*Å*
Maxima						
2240 ± 10	6100	130	0 0086	2 0	0 25	12 0
2300 10	5050	200	0 0086	2 0	0 15	7 8
2810 5	2045	20	0 0230	2 0	0 06	7 2
2870 10	1940	50	0 0230	2 0	0 06	7 8
Minima						
2485 ± 15	222	10	0 0072	2 0	0 08	5 4
2860 10	1910	30	0 0230	2 0	0 06	7 8
Androsterone; sample weight, 150 mg. (1 run)						
Maximum						
2925 ± 10	42 6		1 27	2 0	0 075	10 6
Minimum						
2325 ± 25	6 6		2 50	2 0	0 2	11 2

Δ⁴-Androstenedione-3,17—A pure sample was supplied by Professor Ruzicka.

Cholestenone—The prepared sample was recrystallized twice from acetone.

Cortical Preparation E—A compound ($C_{21}H_{30}O_5$), $[\alpha]_D^{25} = +270°$, from the suprarenal cortex was isolated and supplied to us by Dr. E. C. Kendall (4).

TABLE VI

Absorption Data for Androstenedione-3,17, Cholestenone, and Cortical Preparation E in Ethyl Alcohol

	Molecular α	Maximum deviation, 2 runs	Concentration	Cell thickness	Slits	Spectral range isolated
			Androstenedione			
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maximum						
2408	15,755		0.00280	2.0	0.15	9.5
			Cholestenone			
Maxima						
2420 ± 10	16,300	100	0.0075	1.0	0.11	7.3
3125 20	76		0.184	7.0	0.08	14.5
Minimum						
2850	55		0.184	7.0	0.08	10.2
			Cortical Preparation E			
Maximum						
2370	16,150		0.0076	1.0	0.20	6.0

Absorption data for androstenedione, cholestenone, and the cortical Preparation E appear in Table VI.

Cresols—*o*-, *m*-, and *p*-cresols were repurified from Kahlbaum's reagent quality products by triple distillation (Tables VII and VIII).

Highly purified samples of cholesterol were supplied to us by Dr. Elizabeth M. Koch. Cholesteryl chloride, m.p. 103.5–104.5°, dihydrocholesterol, m.p. 142°, and epidihydrocholesterol, m.p. 184–186°, were prepared, purified, and supplied to us by Dr. T. F. Gallagher.

Spectra

Phenanthrene (*Fig. 1*)—While this spectrum is in good agreement with that obtained by Cook, Hewett, Mayneord, and Roe (5) when wave-length and relative intensity of the bands are considered, the magnitudes of the absorption coefficients do not agree in all respects.

TABLE VII

Absorption Data for Cresols in Ethyl Alcohol

Sample weight, 45 mg.

	Molecular α	Maximum deviation, 2 runs	Concentration	Cell thickness	Slits	Spectral range isolated
			p-Cresol			
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2240 ± 10	6160	60	0.0060	1.0	0.15	7.0
2800 5	1850	10	0.0098	2.0	0.05	5.9
Minimum						
2435 ± 20	79		0.0098	7.0	0.08	5.5
			o-Cresol			
Maximum						
2720 ± 5	1950	40	0.0098	2.0	0.10	10.5
Minimum						
2400 ± 15	108		0.0098	7.0	0.15	5.0
			m-Cresol			
Maxima						
2740 ± 5	1690	30	0.0098	2.0	0.04	4.3
2800 5	1510	20	0.0098	2.0	0.04	4.7
Minima						
2425 ± 10	88		0.0098	7.0	0.05	3.3
2780 5	1460	20	0.0098	2.0	0.04	4.6

Phenanthrenequinone (*Fig. 2*)—The spectrum of this substance has been studied by Marchlewski and Moroz (6). The data given by these workers do not include the bands at 2570, 2650, and 4125 Å. The curve given by them is qualitative in character.

Cholesterol—The absorption spectra of cholesterol samples vary

TABLE VIII

Absorption Data for Cresols in Isooctane

Sample weight, 45 mg.

	Molecular α	Maximum deviation, 2 runs	Concentration	Cell thickness	Slits	Spectral range isolated
			p-Cresol			
Å.			*gm. per l.*	*cm.*	*mm.*	*Å.*
Maxima						
2220 ± 5	4880	150	0.0070	1.0	0.15	7.0
2620	623	3	0.0360	1.0	0.05	4.6
2645	795	1	0.0360	1.0	0.05	4.7
2675	1020	10	0.0360	1.0	0.05	4.9
2695	1350	10	0.0360	1.0	0.05	5.1
2730	1540	20	0.0180	1.0	0.045	4.8
2755	1745	20	0.0180	1.0	0.045	5.0
2785	2090	40	0.0180	1.0	0.045	5.2
2820	1275	10	0.0360	1.0	0.05	6.1
2850	1850	10	0.0360	1.0	0.06	7.7
Minima						
2425 ± 15	90		0.0090	7.0	0.08	5.3
2710 3	1220	10	0.0360	1.0	0.05	5.2
2740 3	1440	10	0.0180	1.0	0.045	4.8
2770 3	1630	10	0.0180	1.0	0.045	5.2
2815 3	1240	20	0.0360	1.0	0.05	6.1
2830 3	1180	30	0.0360	1.0	0.05	6.2
			o-Cresol			
Maxima						
2200	2930	200	0.0100	1.0	0.20	8.8
2710 ± 3	1850	15	0.0180	1.0	0.05	5.2
2775 5	1710	10	0.0180	1.0	0.05	5.7
Minima						
2375 ± 15	87		0.0100	7.0	0.12	7.2
2750 3	1350	10	0.0180	1.0	0.05	5.5
			m-Cresol			
Maxima						
2200	3980	100	0.0070	1.0	0.20	8.8
2720 ± 5	1700	10	0.0190	1.0	0.04	4.2
2790 5	1800	30	0.0190	1.0	0.05	5.8
Minima						
2400 ± 15	80		0.0130	7.0	0.10	6.3
2760 5	1030	30	0.0190	1.0	0.05	5.6

considerably, depending upon purification treatment. Extremely pure samples absorb so little in the ultraviolet region above 2200 Å., that it is difficult to determine whether the observed absorption (molecular α less than 2.0) is due to cholesterol or to minute amounts of impurity. It was apparent that drying temperatures in the neighborhood of 50° cause sufficient decomposition to affect the absorption spectrum. Illumination of the material must also be avoided.

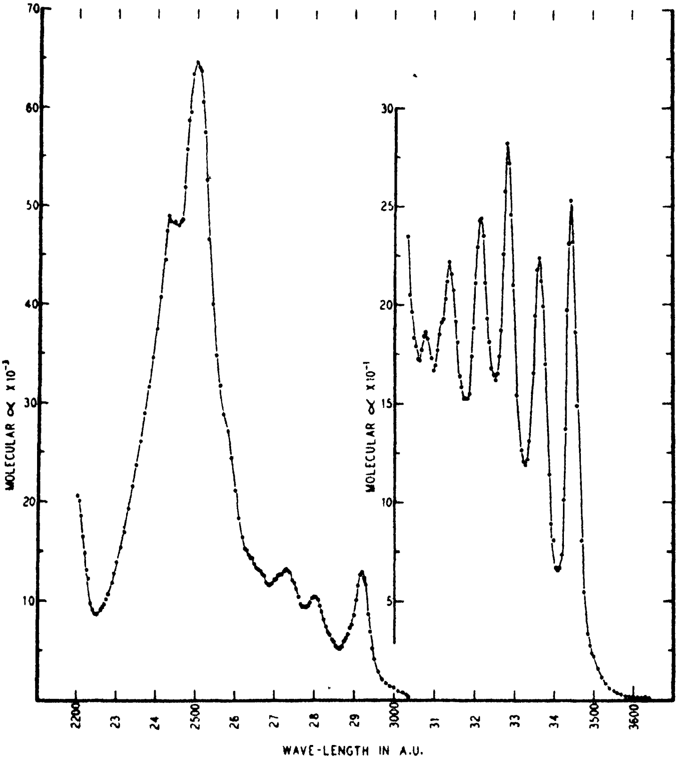

FIG. 1. Absorption spectrum of phenanthrene in ethyl alcohol

Bills, Honeywell, and MacNair (7) demonstrated that samples of cholesterol exhibit weak general absorption in the ultraviolet region when properly purified by the removal of ergosterol. This fact has been confirmed by the work of Koch, Koch, and Lemon

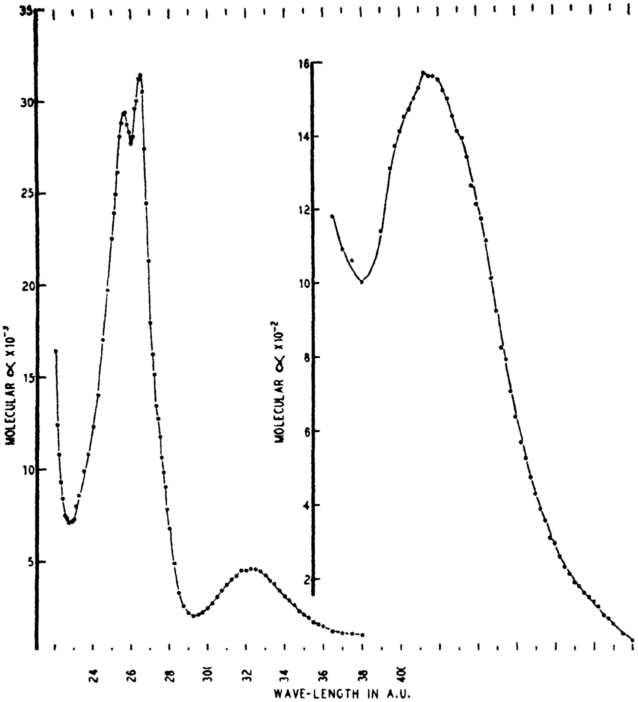

FIG. 2. Absorption spectrum of phenanthrenequinone in ethyl alcohol

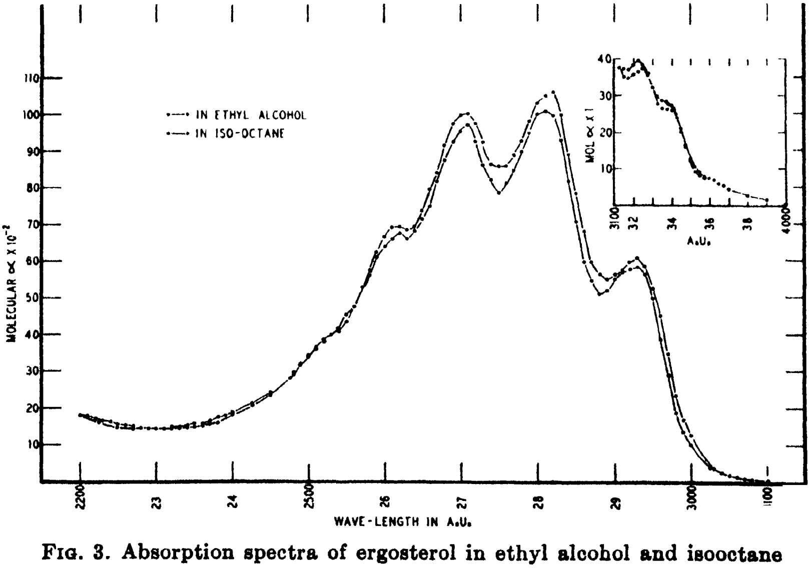

FIG. 3. Absorption spectra of ergosterol in ethyl alcohol and isooctane

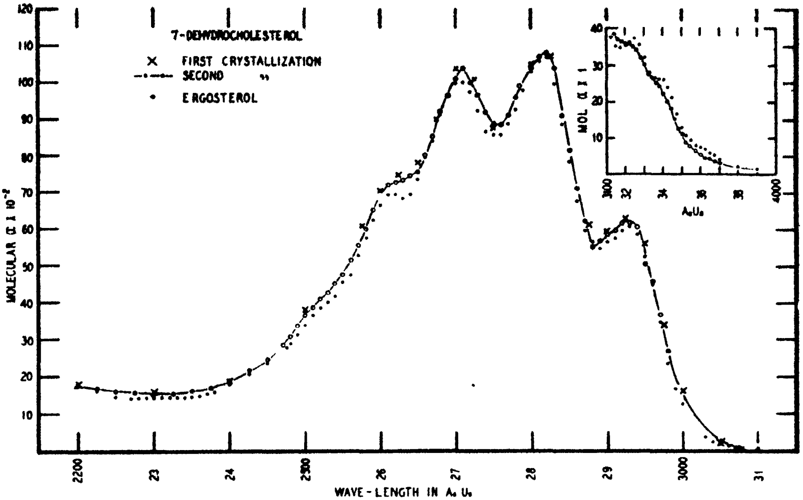

Fig. 4. Absorption spectrum of 7-dehydrocholesterol and ergosterol in ethyl alcohol solution.

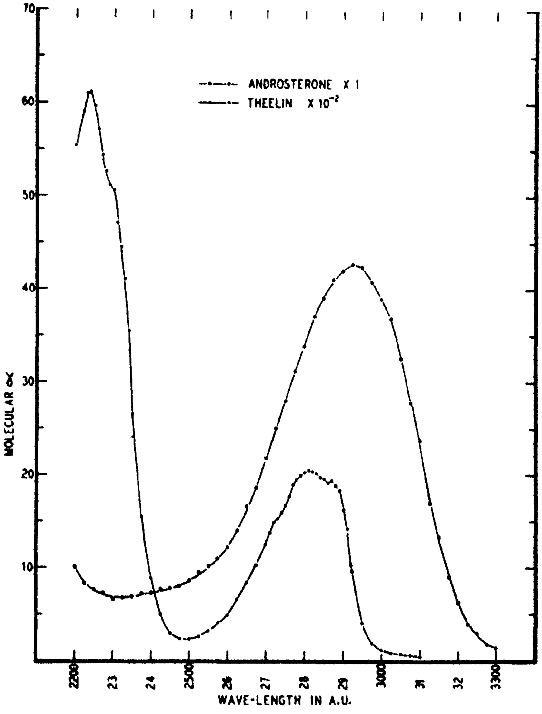

Fig. 5. Absorption spectra or theelin and androsterone in ethyl alcohol

(8) and by Hathaway and Koch (9). Our results are in agreement with those of these workers.

Experiments carried out in this laboratory have shown that cholesteryl chloride, dihydrocholesterol, and epidihydrocholesterol absorb ultraviolet light to about the same extent as pure cholesterol. In this connection the work of Heilbron, Morton, and Sexton (10) is interesting. They found low general absorption for the compounds cholesterol, cholesteryl chloride, cholesteryl acetate, cholestene, ψ-cholestene, and dicholesteryl ether.

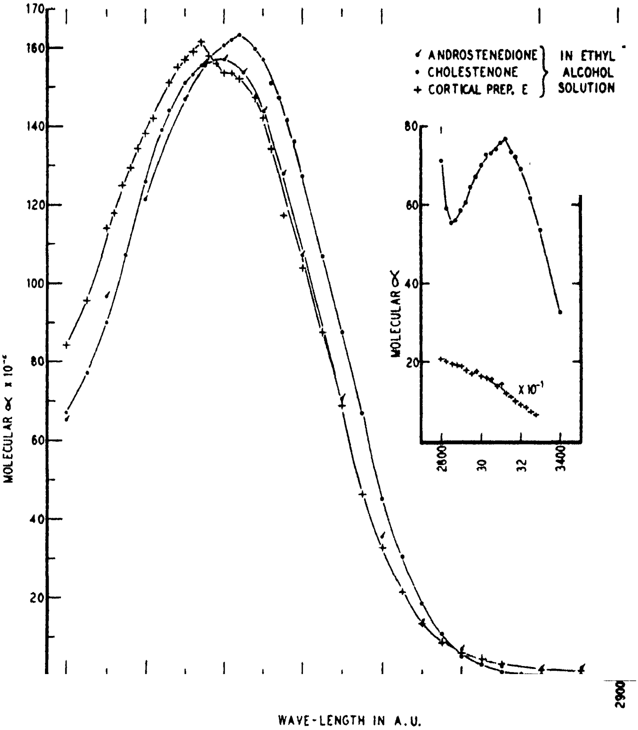

FIG. 6. Absorption spectra of cholestenone, androstenedione-3,17, and cortical Preparation E in ethyl alcohol.

Ergosterol (*Fig. 3*)—This substance has been studied recently by Windaus, Lettre, and Schenck (3). The spectra in isooctane and ethyl alcohol are but slightly different. New bands are here reported at 3225 to 3250, and 3375 Å. The band at 2520 Å. is quite distinct as compared with the shoulder on previous curves of ergosterol. These bands were very probably not detected before because of photographic difficulties, the use of too dilute solutions, or too short absorption cells.

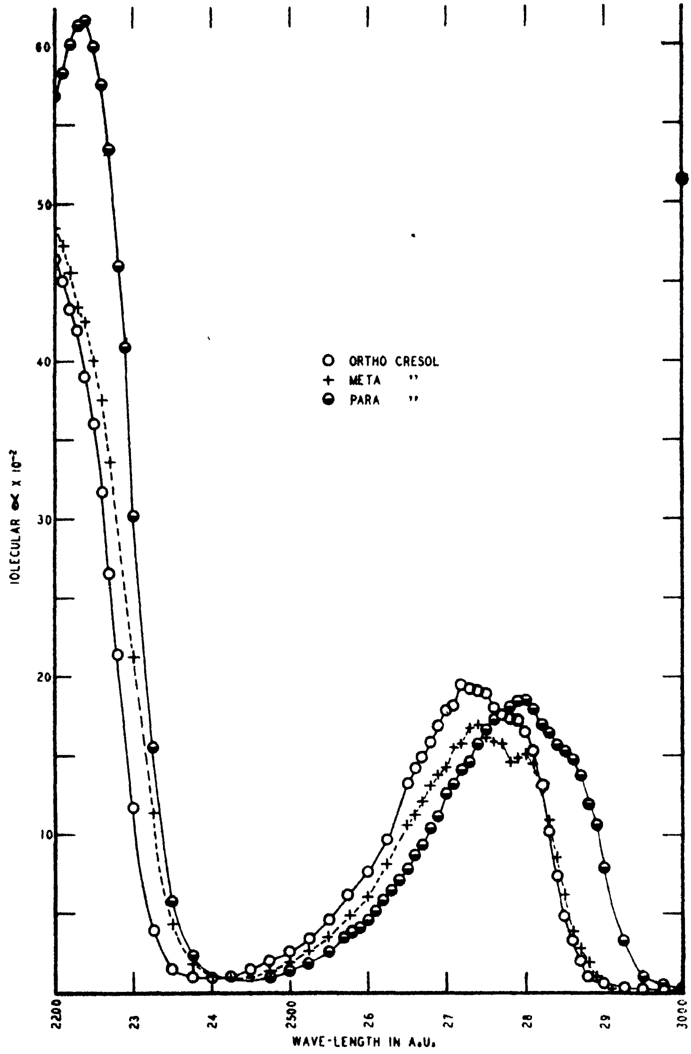

FIG. 7. Absorption spectra of *o*-, *m*-, and *p*-cresols in ethyl alcohol

7-Dehydrocholesterol (*Fig. 4*)—This compound was found to exhibit almost the same absorption curve as ergosterol, which fact was pointed out by Windaus, Lettre, and Schenck (3). Slight differences in magnitude, however, cause the bands at 2520, 2625, 3210, and 3360 Å. to be less distinct than the corresponding bands of ergosterol.

Theelin (*Fig. 5*)—The spectra of theelin (estrone) and theelol (estriol) were found to be practically identical. The curve drawn through the solid circles (Fig. 5) represents the spectra of these substances. This spectrum is in qualitative agreement with the work of Butenandt and Stormer (11) and with the recent work of Callow (12).

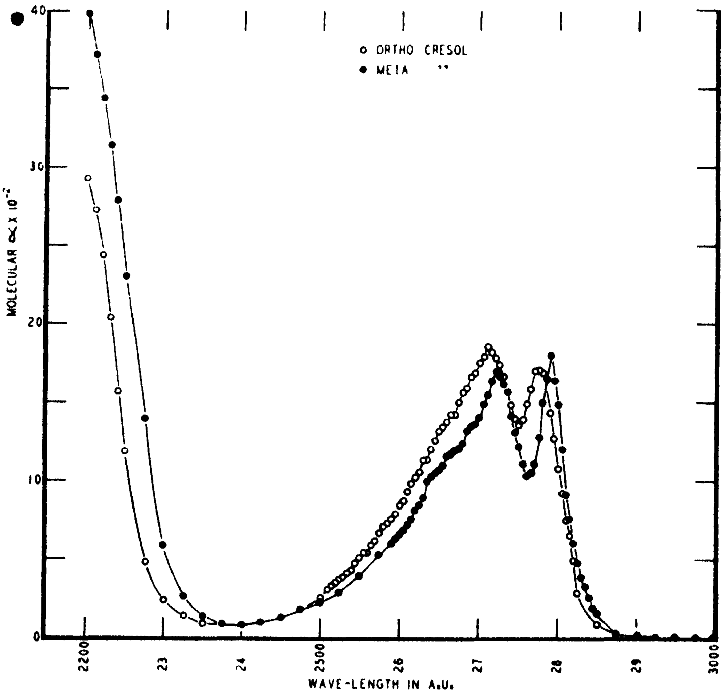

FIG. 8. Absorption spectra of *o*- and *m*-cresols in isooctane

Androsterone-3,17 (*Fig. 5*)—The spectrum of androsterone-3,17 exhibits a relatively broad, symmetrical band with a maximum occurring at 2925 Å. The magnitude of the absorption coefficient

($\alpha = 42.6$) at this maximum (Fig. 5, curve drawn through the clear circles) is significantly low when compared with the values obtained for the α,β-unsaturated ketones shown in Fig. 6.

Δ^4-*Androstenedione-3, 17* (*Fig. 6*)—The absorption spectrum of this substance has been studied by Butenandt and Kudszus (13). The spectrum published by these workers shows a maximum at 2350 Å., as determined in chloroform solution. A small band appearing at 2775 Å. in that solvent is absent in the spectrum presented here, as determined in ethyl alcohol solution.

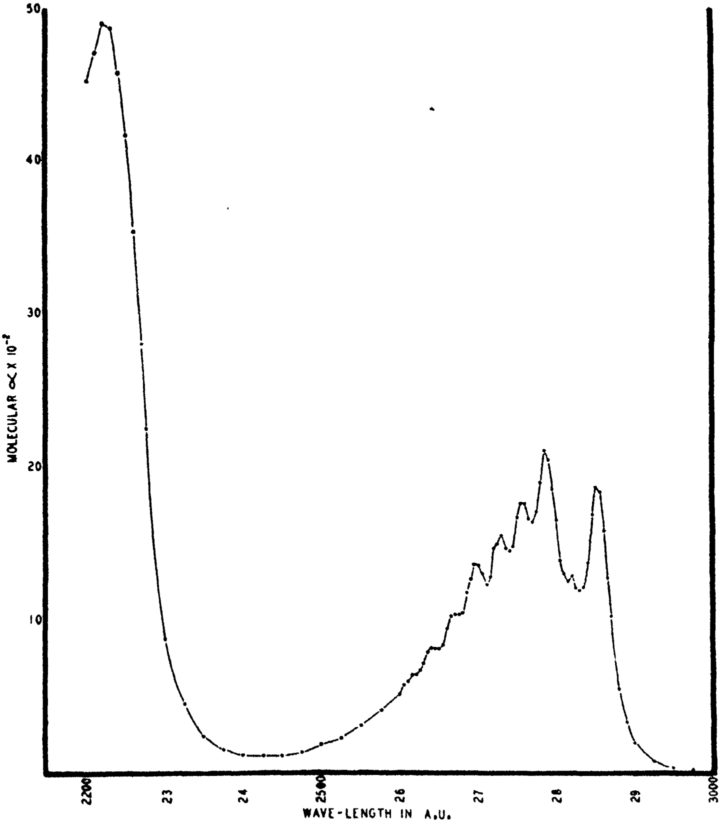

Fig. 9. Absorption spectrum of *p*-cresol in isooctane

Cholestenone (*Fig. 6*)—Cholestenone has been studied by Heilbron, Morton, and Sexton (10) and by Menschick, Page, and Bossert (14) who showed that absorption at 2400 Å. is characteristic for α,β-unsaturated ketone compounds of this type. The curve presented here is in qualitative agreement with that of those workers.

Cortical Preparation E (*Fig. 6*)—This compound is identical with the Compound E described by Mason, Myers, and Kendall (4). Its spectrum is strikingly similar to the spectra of androstenedione and cholestenone. This fact has been considered an indication that the basic structure of this compound must include the α,β-unsaturated ketone grouping present in those substances.

Cresols (*Figs. 7 to 9*)—The spectra of *o*-, *m*-, and *p*-cresols were studied in hexane solution by Wolf and Herold (15). Their data are in fair agreement with those reported here for these isomers in isooctane solution. The absorption bands of these substances exhibit increased sharpness and more detail in isooctane solution than in the more polar solvent ethyl alcohol. The three isomers may be distinguished from each other with ease in either solvent.

DISCUSSION

A logical application of the results and technique of the spectrophotoelectric method employed to determine the spectra here presented would be the correlation of absorption spectra of physiologically important substances with biological activity. A simple calculation based upon the specific absorption coefficients of the hormones theelin (estrone) and androsterone and their biologically active potencies in urine shows that these substances do not occur in sufficient concentration to make spectroscopic analysis directly practical. Unfortunately, the striking similarity which exists between the spectra of theelin (estrone), estradiol, and theelol (estriol) and *p*-cresol further complicates any such analysis. Attempts were made in this laboratory to assay urine concentrates for female hormone activity by the spectroscopic method. A general correlation between estrogenic activity (rat units per cc. of original urine) and the absorption at 2800 Å. was found, but the absorption was due to the presence of *p*-cresol in relatively high concentrations. *p*-Cresol was isolated from several urine concentrates and positively identified by its absorption spectrum.

A more fruitful application of this spectroscopic technique is in the identification of basic structure types. Several instances of similar absorption spectra arising from identity of basic molecular structure are apparent within the group of spectra reported here. Ergosterol and 7-dehydrocholesterol have been shown to have the same conjugated structure in Ring 2 of the cyclopentenophenanthrene nucleus. Cholestenone and androstenedione-3,17 have the same α,β-unsaturated ketone structure present in Ring 1. It is apparent that the compound isolated from the suprarenal cortex (Compound E) possesses a spectrum which suggests that it too may have ketonic oxygen in position (3) and a double bond between carbon atoms (4) and (5) in the sterol nucleus. The structural formula proposed by Kendall, Mason, and Myers (16) is very probably the correct one, inasmuch as it involves a doubly conjugated system. Such a structure would probably give a greater molecular absorption coefficient and would show an absorption maximum somewhat toward the longer wave-lengths.

We wish to express our appreciation to Dr. Fred C. Koch for the purification of the theelin; to Dr. Elizabeth M. Koch for the preparation and purification of the 7-dehydrocholesterol and purification of the ergosterol; and to Dr. Thomas F. Gallagher for the preparation and purification of the cholestenone used in these experiments.

BIBLIOGRAPHY

1. Hogness, T. R., Zscheile, F. P., and Sidwell, A. E., Jr., *J. Physic. Chem.*, **41**, 379 (1937).
2 Cohen, F. L., and Cormier, U., *J. Am. Chem. Soc.*, **52**, 4363 (1930).
3. Windaus, A., Lettre, H., and Schenck, F., *Ann. Chem.*, **520**, 98 (1936).
4. Mason, H. L., Myers, C. S., and Kendall, E. C., *J. Biol. Chem.*, **114**, 613 (1936).
5. Cook, J. W., Hewett, C. L., Mayneord, W. V., and Roe, E., *J. Chem. Soc.*, 1727 (1934).
6. Marchlewski, L., and Moroz, A., *Bull. Soc. chim.*, **35**, 473 (1924).
7. Bills, C. E., Honeywell, E. M., and MacNair, W. A., *J. Biol. Chem.*, **76**, 251 (1928).
8. Koch, E. M., Koch, F. C., and Lemon, H. B., *J. Biol. Chem.*, **85**, 159 (1929–30).

9. Hathaway, M. L., and Koch, F. C., *J. Biol. Chem.*, **108**, 773 (1935).
10. Heilbron, I. M., Morton, R. A., and Sexton, W. A., *J. Chem. Soc.*, 47 (1928).
11. Butenandt, A., and Stormer, I., *Z. physiol. Chem.*, **208**, 129 (1932).
12. Callow, R. K., *Biochem. J.*, **30**, 906 (1936).
13. Butenandt, A., and Kudszus, H., *Z. physiol. Chem.*, **237**, 75 (1935).
14. Menschick, W., Page, I. H., and Bossert, K., *Ann. Chem.*, **495**, 225 (1932).
15. Wolf, W. L., and Herold, W., *Z. physik. Chem.*, *Abt. B*, **13**, 229 (1931).
16. Kendall, E. C., Mason, H. W., and Myers, C. S., *Proc. Staff Meetings Mayo Clin.*, **11**, 351 (1936).

A MICROMETHOD FOR THE DETERMINATION OF GELATIN AND A STUDY OF THE COLLAGEN CONTENT OF MUSCLES FROM NORMAL AND DYSTROPHIC RABBITS

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In the course of our investigation of nutritional muscle dystrophy the need for some chemical method for determining the degree of dystrophy was soon recognized. Since in the dystrophying muscle there is a progressive accumulation of fibrous tissue, it seemed to us that a microdetermination of the collagen content, in the form of gelatin nitrogen, would furnish the necessary clue for such an estimation.

Method

In recent years two procedures have been suggested for the determination of collagen as gelatin N. Mitchell, Zimmerman, and Hamilton (4) worked out a method requiring a 25 to 100 gm. sample of meat. The meat is macerated in a ball mill, filtered through a 40 mesh sieve, and the residue is thoroughly washed with water. The washed residue, suspended in water, is then autoclaved for 2 hours at 16 to 18 pounds pressure. The filtrate from the autoclaved residue, together with the washings, is decanted into a volumetric flask, and the N determination is made on an aliquot portion of this solution containing all the gelatin.

Farkas (1) proposed a method for the analysis of gelatin-protein mixtures based upon the fact that gelatin-picrate is soluble at 40°, and could thus be separated from other precipitated proteins at that temperature. By making the precipitation with picric acid at 40° and subsequent extraction of the precipitate with warm water all of the gelatin in the original material could be removed. When the solution has cooled, the gelatin is precipitated and is washed free from picric acid with Brücke's reagent (10 per cent KI saturated with HgI_2) on the filter. The washed residue is then digested and analyzed for N. Than (9) utilized this procedure in determining the collagen content of 0.5 gm. of dried meat.

Obviously, the Mitchell method cannot be used for small amounts of tissue, while the Farkas-Than method is very tedious and time-consuming, and not well suited for routine work.

Stoke's method (8), accepted by the Association of Agricultural Chemists for the determination of gelatin in milk products, employs $Hg(NO_3)_2$, which precipitates all proteins except gelatin. To the filtrate, containing the gelatin in solution, picric acid is added to precipitate the gelatin. Our objection to the Farkas-Than procedure holds equally well for this method. Jacobs and Jaffe (2) modified this official method by employing basic lead nitrate as the reagent to precipitate various proteins and by subjecting the gelatin-containing filtrate to a preliminary treatment with activated charcoal, which adsorbs pseudogelatins and proteans. The gelatin contained in the final filtrate was then precipitated with tannic acid.

In developing our procedure which is quick, simple, and applicable to very small quantities of material we availed ourselves of the best features of these various methods: minced muscle is dried and powdered, the collagen is converted to gelatin by autoclaving, the gelatin is extracted with hot water and is, finally, precipitated with tannic acid.

Procedure

The muscle to be analyzed is freed from adhering tissue, minced, and a weighed amount is dehydrated with acetone. For this purpose the minced tissue in a weighing bottle is completely covered with acetone which is allowed to evaporate spontaneously at room temperature. The material is then dried to constant weight in a vacuum desiccator and ground to a fine powder. A 100 to 200 mg. sample of the powder (corresponding to about 0.5 to 1 gm. of fresh tissue) is weighed into a small centrifuge tube, suspended in about 4 cc. of water, and autoclaved for 2 hours at 15 to 20 pounds pressure. After cooling, the tube is centrifuged and the liquid is poured off into a large centrifuge tube. The residue in the original tube is extracted three times with small amounts of hot water, centrifuged, and the washings also transferred to the large tube, which now contains all the gelatin. The solution is slightly acidified, 5 cc. of a 5 per cent tannic acid reagent added, and the tube set in the ice box for at least 1 hour, whereupon the gelatin is quantitatively precipitated. The precipitated gelatin is centrifuged off and is dissolved by heating gently with 1 cc. of 2 N NaOH and 5 to 10 cc. of water. The gelatin solution is transferred to a small Kjeldahl flask, to which are also added the washings; the contents are neutralized with 1 cc. of 2 N H_2SO_4 and digested, with 2 cc. of a 1:1 H_2SO_4 solution and H_2O_2. The digested material after cooling is transferred to a 100 cc. volumetric flask and diluted to volume. The N determination is carried out on aliquot portions of this solution, containing 0.5 to 1.0 mg. of N, by direct nesslerization.

The total N of the muscle powder was determined by a procedure which we described elsewhere (7).

Analyses of Isoelectric Gelatin

To test the accuracy of this micromethod we analyzed isoelectric gelatin which we prepared according to Loeb's direction (3). Our preparation contained 17.2 per cent of N. Using a 0.21 per cent solution of this isoelectric gelatin, we determined the N of the tannic acid precipitate made either directly or after a preliminary autoclaving of the gelatin solution. The procedure was exactly as previously described. In both instances the N content was found to be 17.2 per cent as of the original preparation.

Experiments with the isoelectric gelatin solution were made to determine the time necessary to allow the tannic acid precipitation to become quantitative, and we found that 1 hour in the ice box secures quantitative precipitation of the gelatin.

Isoelectric gelatin was added in known amounts to muscle powder, the gelatin content of which had been determined. Analyses have proved that added gelatin was quantitatively recovered.

Finally, we studied the effect of various non-protein nitrogenous substances on the gelatin determination. We added creatinine, uric acid, or urea to samples of the isoelectric gelatin solution, and analyzed for gelatin following all the steps of the determination as previously described. In every instance the gelatin determination was quantitative.

The experiments with the isoelectric gelatin of known N content show, therefore, that the tannic acid is capable of precipitating small amounts of gelatin quantitatively, that the gelatin itself is not affected by autoclaving either alone or together with muscle powder, and that the presence of various non-protein nitrogenous substances does not interfere with the quantitative gelatin determination by tannic acid precipitation.

Collagen Content of Normal and Dystrophic Muscles

In this study of the collagen content of muscles from normal rabbits and from rabbits made dystrophic by special dietary treatment our chief aim was to trace the development of the disease. The animals used for this experiment were all practically of the same age (between 30 and 40 days old) but owing to the large number required they belonged to six different litters. As was pointed out in a previous paper (5) rabbits on the Goettsch-Pappenheimer Diet 13 become dystrophic in about 3 to 6 weeks and usually die shortly after the onset of the disease, unless furnished with foodstuffs which secure recovery. In these experiments we employed our modified Diet 313, in which treatment with superoxol is substituted for the ethereal $FeCl_3$ treatment and which is just as effective. The body weight curve of rabbits on this dystrophy-producing diet is, for a time, the same as of the control animals. After a while, however, the daily gains become irregular and alternate with losses. We designate this as the *period of wavering weight.* Finally, an abrupt drop in the body weight occurs, and the condition of the animal progresses rapidly to death. We designate the sharp break in the weight curve as the *critical point.* Usually, a few days beyond this point, the condition of the animals is so far advanced that it is no longer possible to save them by dietary treatment. A number of interesting and critical changes occur at this time in the metabolism (6).

Our object was to follow the changes in collagen content of the muscles from the time the rabbits have been placed on this Diet 313 until they develop definite signs of dystrophy. Since no two animals behave exactly alike, the disease developing at a variable and totally unpredictable rate in each, it was not possible to so select animals for the study of their muscles as to obtain a continuous series of stages. About 50 young rabbits were separated into a control and experimental group, care being taken that they represented approximately the same distribution as regard litters and initial weights. The experimental rabbits were given our Diet 313, while the control animals received this diet supplemented with wheat germ, which we have shown to be an excellent preventive of the disease. Every animal was weighed daily and individual weight charts were kept. At different times rabbits were selected from both groups, and usually three muscles (the left gastroenemius, left biceps femoris, and left triceps brachii) were taken for the collagen determinations. The material was analyzed in accordance with the procedure previously described. The results of these analyses are presented in Table I.

The control rabbits were killed at different times between the age of 36 and 79 days. An examination of the total and gelatin N content of the three sets of muscles shows that this is apparently not affected by age. The total N of the gastrocnemius, biceps, and triceps was, on the average, 13.92, 13.93, and 13.64 per cent, respectively. These values are calculated on the basis of the dry weight of the muscles. The total gelatin N for the corresponding muscles was 1.91, 1.47, and 1.77 per cent. The proportion of total muscle N in the form of collagen N in the three muscles was 13.7, 10.5, and 13.0 per cent, respectively.

It is important to point out that the muscles with the highest collagen content show the smallest range of variation. Thus, the gastrocnemius has an average ratio of gelatin N to total N of 13.7 per cent, with a range between maximum and minimum values of 2 per cent. The triceps muscle, with an average of 13.0 per cent, has a range of variation of 4 per cent, and the biceps, with an average of 10.0 per cent, has a range of 5 per cent (if one exceptionally high value is not included in the calculation, otherwise the average is 10.5 per cent and the range is 7 per cent).

Before describing the condition found in their litter mates in the course of development of dystrophy, we shall make brief reference to analyses made on the same muscles from several animals, belonging to other experimental series, which were all in a more or less advanced stage of dystrophy. When the average values (13.34, 13.77, and 13.44 per cent) are examined, it will be

Table I

Collagen Content of Normal and Dystrophic Rabbit Muscles (on Basis of Dry Weight)

Rabbit No.	Age at death	Days on Diet 313	Gastrocnemius			Biceps femoris			Triceps brachii			Remarks
			Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	
	days		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		
158	36					13.50	1.65	12.2	13.40	1.79	13.4	Control; Diet 313 + wheat germ
137	37					14.04	1.67	11.9	13.68	1.61	11.8	“ “
122	41								13.53	1.81	13.4	“ “
120	47		14.04	2.01	14.3	14.37	2.08	14.5	14.03	1.85	13.2	“ “
131	54		13.90	2.02	14.5	13.65	1.44	10.5	13.28	2.00	15.1	“ “
149	56		14.20	1.82	12.8	13.90	1.22	8.8	13.79	1.56	11.3	“ “
136	69		13.68	2.00	14.6	13.42	0.98	7.4	14.04	1.77	12.6	“ “
127	75		13.75	1.73	12.6	14.23	1.42	10.0	13.01	1.79	13.8	“ “
147	79		13.96	1.89	13.5	14.33	1.28	8.9	13.96	1.79	12.8	“ “
Average..........			13.92	1.91	13.7	13.93	1.47	10.5	13.64	1.77	13.0	
128	40	5	13.33	1.79	13.4	13.68	1.39	10.2	14.03	1.52	10.8	Apparently normal
123	51	10	13.40	2.22	16.6	13.54	1.31	9.7	13.90	1.47	10.6	“ “
129	52	17	14.04	2.27	16.2	13.50	1.48	11.0	13.47	2.22	16.5	“ “
143	54	18	13.90	2.37	17.1	13.53	1.89	14.0	13.96	1.92	13.8	“ “
124	59	18	13.90	1.79	12.9	13.33	1.86	14.0	13.60	2.17	16.0	“ “
151	59	22				13.75	2.08	15.1	13.73	2.08	15.1	“ “
126	63	22	13.60	1.87	13.7	13.64	1.20	8.8	13.13	1.77	13.5	“ “

141	61	25	13.75	2.00	14.5	13.96	1.66	11.9	13.33	2.57	19.3	Apparently normal
148	62	25				13.88	1.67	12.0	13.90	2.08	15.0	Slight dystrophy
144	62	26				13.81	1.79	13.0	13.62	2.94	21.6	Apparently normal
132	63	28	13.60	1.79	13.2	14.70	1.75	11.9	13.33	2.03	15.2	Wavering in weight
152	70	32				14.33	2.35	16.4	13.61	2.78	20.4	Slight dystrophy
135	72	35				14.70	1.60	10.9	13.79	3.03	22.0	Wavering in weight
138	72	36				13.60	2.56	18.8	13.40	3.23	24.1	Slight dystrophy
134	73	36				13.83	2.73	19.7	13.33	3.33	25.0	Definite dystrophy
121	79	39				14.33	1.54	10.7	13.53	2.17	16.0	Wavering in weight
154	71	32				13.68	3.38	24.7	13.61	2.94	21.6	Definite dystrophy
142	76	40				12.96	2.32	17.9	13.54	2.62	19.3	Slight dystrophy
139	83	46				13.50	3.23	23.9	13.44	3.03	22.5	Definite dystrophy
130	90	46				13.47	1.91	14.2	13.44	3.17	23.6	9 days on curative diet; recovering
153	96	46				13.40	2.08	15.5	13.53	2.57	19.0	20 days on curative diet; recovering
119	79		13.20	4.31	32.7	13.37	3.28	24.5				Definite dystrophy
101	114		13.40	4.24	31.6	13.56	2.65	19.5	13.47	4.63	34.4	" "
114	95		13.33	3.91	29.3	13.40	2.85	21.3	13.33	4.43	33.2	" "
117	110		13.30	4.16	31.3	14.31	4.17	29.1	13.33	4.51	33.8	" "
107	116		13.47	4.95	36.7	14.19	2.78	19.6	13.50	3.32	24.6	" "
154	70								13.55	3.33	24.6	" "
Average..........			13.34	4.31	32.3	13.77	3.15	22.8	13.44	4.04	30.1	

noted that the total N content of the seven diseased rabbits was somewhat decreased, but the diminution, except in the case of the gastrocnemius, is not significant. The collagen content, however, is from 2 to 2.5 times as great, the per cent of total N as gelatin N increasing to 22.8 to 32.3 per cent. In other words, the collagen, which makes up about one-fiftieth to one-seventieth of the dry weight of normal muscles, is about one-twentieth to one-thirtieth of the dystrophic muscles. In the group of muscles studied the largest increase is found in the gastrocnemius and the smallest increase in the biceps femoris, and this also corresponds to the general collagen content of these three muscles.

At various intervals we have analyzed the muscles of the animals which were on the dystrophy-producing Diet 313 from 5 to 46 days (age 40 to 83 days). For about 25 days there are no outward signs by which one can detect the onset or progress of dystrophy, and such animals are designated as "apparently normal." The first symptoms of the onset of dystrophy, apart from the wavering weight curve, is the peculiar posture and inability of the animal to right itself, and, depending upon the severity of these outward signs, the condition is described as slight or definite dystrophy. Unfortunately, owing to the fact that the muscles were used also for other investigations, we omitted the gastrocnemius in animals in the more advanced stage of the disease. Of the eight rabbits whose gastrocnemii were analyzed within the first 25 days on Diet 313, five have collagen values entirely within the normal range but three rabbits, though outwardly they appear entirely normal, show a slight but definite increase in the collagen content between the 2nd and 3rd week.

The triceps brachii was analyzed in all of the nineteen experimental rabbits. Here, likewise, for about 4 weeks the collagen values are practically within the normal range. Soon after that, however, there is a marked rise in collagen content. It is rather interesting to point out that of the five animals examined within the 5th week two animals, with no outward signs of dystrophy, showed a large increase in collagen content, and one was within the normal range. But two animals, already affected, have a collagen content within the uppermost normal range. Of course, this may mean that the latter have started at the lowest normal range, while the former had started at the highest normal range and that actually both groups of animals have a markedly increased collagen content. Another point in this connection which merits attention is the fact that a considerable dystrophic change in the muscle may take place before the outward signs of dystrophy become manifest. However, between the 5th and 6th week, when the outward signs are present and the condition increases in severity, there is a very abrupt and rapid development of collagen in this muscle.

The results with the biceps femoris are essentially the same. We have already mentioned that the normal range of variation of the collagen content of this muscle is large. We find, therefore, that four out of thirteen rabbits examined with outward manifestations still show a collagen which falls within the upper range of normal variation, but four animals, without any external signs, show a small but unmistakable increase in collagen. By the end of about 5 weeks not only does the disease develop rapidly, but the collagen content of this muscle, like that of other muscles examined, increases rather abruptly and quickly.

Two animals of this series, after they had developed definite dystrophy, were changed to a curative diet (Diet 313 + wheat germ). They were sacrificed at the end of 9 and of 20 days on this diet. They had both recovered from the outward signs of the disease. The collagen content of the biceps had almost returned to normal, while the recuperative changes in the triceps muscle are decidedly slower but nevertheless progressive.

SUMMARY

A method is described for the microdetermination of collagen as gelatin N, which depends upon the conversion of collagen to gelatin by autoclaving 100 to 200 mg. of substance dried by means of acetone. The gelatin is precipitated by means of tannic acid; the precipitate is dissolved in dilute alkali and digested with H_2SO_4 and H_2O_2. The digest is made up to a known volume and aliquot portions are directly nesslerized. The quantities are so adjusted that the aliquots contain 0.5 to 1.0 mg. of N.

The collagen content of the gastrocnemius, biceps femoris, and triceps brachii was determined in a number of growing rabbits. The collagen content, as well as the per cent of total N in the form of gelatin N, was found to be independent of the age of the animals.

The average per cent of total N in the form of gelatin N is greatest for the gastrocnemius and smallest for the biceps, while the range of variations is smallest for the gastrocnemius and greatest for the biceps. Rabbits in which nutritional muscle dystrophy has reached an advanced condition have 2 to 2.5 times as much collagen in the muscles as the control animals.

In the course of the development of the disease definite changes in the collagen content usually begin to appear when the animals have been on the dystrophy-producing Diet 313 about 3 weeks and become quite marked at the time the critical point is reached. The rather rapid development of the fibrous tissue in the muscles thus coincides with the general metabolic reaction at that period. When the animals are cured of the dystrophy, the collagen content of the muscles regresses. The return to the normal condition was much more rapid in the biceps femoris than in the triceps brachii in these animals.

The collagen content may be markedly increased even before the outward signs of dystrophy become apparent. Different muscles of the same animal are apparently not affected to the same degree. However, the collagen determination may be used as a criterion of the early onset of the disease only in the case of a muscle like the gastrocnemius, whose range of normal variation in collagen content is very limited.

BIBLIOGRAPHY

1. Farkas, G., *Biochem. Z.*, **264**, 361 (1933).
2. Jacobs, M. B., and Jaffe, L., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 418 (1932).
3. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 35 (1922).
4. Mitchell, H. H., Zimmerman, R. L., and Hamilton, T. S., *J. Biol. Chem.*, **71**, 379 (1926-27).
5. Morgulis, S., and Spencer, H. C., *J. Nutrition*, **11**, 573 (1936).
6. Morgulis, S., and Spencer, H. C., *J. Nutrition*, **12**, 173, 191 (1936).
7. Morgulis, S., and Spencer, H. C., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 330 (1936).
8. Stokes, A. W., *Analyst*, **22**, 320 (1897).
9. Than, F., *Biochem. Z.*, **264**, 367 (1933).

ANAEROBIC ULTRAFILTRATION

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A method has been devised for ultrafiltration of small amounts of serum without exposure to air. The serum is introduced over mercury, pressure is applied with a column of mercury, and filtration proceeds across a cellophane membrane, the ultrafiltrate displacing mercury from the receiving chamber.

Apparatus

Two flattened semispherical Pyrex glass chambers 4 cm. in diameter and 1.0 to 1.8 cm. deep (Figs. 1 and 2), each with two capillary glass tubes led off from opposite positions on the circumference, and with the flat side ground perfectly smooth and pierced with a hole, *M*, 2.5 cm. in diameter, are held together by the rubber-protected jaws, *J*, *J'*, of a clamp. The chambers are drawn from Pyrex glass tubing 3.75 cm. in diameter and 1.5 mm. thick. They may be obtained from F. Pierce Noble, 110 Benham Street, Hamden, Connecticut. A Bunsen clamp with effective jaw opening of 6.4 cm. is used.

Sections of rubber tubing, *R*, *R'*, *E*, *E'*, $\frac{1}{8}$ inch in inside diameter with walls $\frac{1}{6}$ inch thick are used for connections. *R* and *R'* are supplied with pinch-cocks, *P* and *P'*.

Cellophane (du Pont, No. 300) (Fig. 2, *M*) cut into sections approximately 4 cm. square and soaked for at least an hour and not longer than 7 days in several changes of distilled water is clamped between the glass cells.

To the tubing *B* and *B'* two small leveling bulbs are attached by sections of thin walled rubber tubing, *L* and *L'*, approximately 60 and 32 cm. long respectively. The tubes, *L* and *L'*, are supplied near *B* and *B'* with Mohr pinch-cocks, not shown in the figures. The bulbs are filled with mercury.

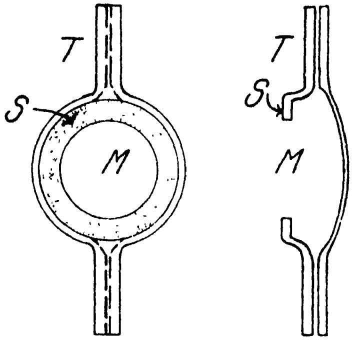

FIG. 1

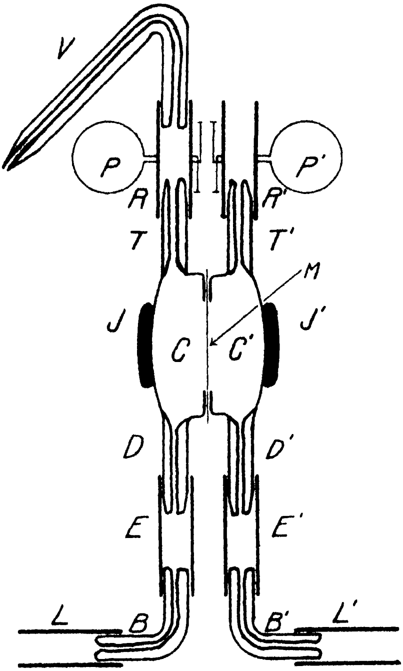

FIG. 2

FIG. 1. Chamber viewed from flat surface and in longitudinal cross-section. *T*, tubing 2 cm. long with 1 mm. capillary bore; *M*, opening 2.5 cm. in diameter in flattened surface; *S*, smoothly ground surface 0.5 cm. wide.

FIG. 2. Apparatus ready for introduction of serum. Both chambers and the tube *V* have been filled with mercury from the leveling bulbs attached to the chambers through the rubber tubing *L* and *L'*. The former, *L*, is then lowered to the table below the chambers, the end of the tube *V* introduced into the serum, and the pinch-cock *P* released until the serum has been drawn into the chamber. *R* and *R'*, rubber tubing; *T* and *T'*, glass tubing 2 cm. long with 1 mm. capillary bore; *M*, opening 2.5 cm. in diameter in flattened surface; *J* and *J'*, rubber-protected jaws of a Bunsen clamp; *C* and *C'*, chambers; *D* and *D'*, glass tubing; *E* and *E'*, rubber tubing; *B* and *B'*, glass tubing; *L* and *L'*, thin walled rubber tubing leading to leveling bulbs.

Procedure

The short sections of rubber tubing are attached to the capillary outlets of the chambers. A square of cellophane is taken from distilled water, shaken to remove excess water, and laid across the flat surface of one chamber while still wet. The second chamber is then placed in apposition and the two are fastened together with a rubber band. The chambers are then placed in the rubber-protected jaws of the Bunsen clamp which are pressed together as firmly as possible between the fingers and palm of one hand while the screw is tightened to hold the jaws at this point without applying any considerable force to the screw. Air is drawn through each chamber for 2 minutes to remove the small amount of water left on the membrane. The connecting tubes and leveling bulbs are then attached as shown in Fig. 2.

The chambers and tube *V* are filled with mercury from the leveling bulbs, *L*, *L′*, and sealed above with pinch-cocks, *P*, *P′*. The lower openings, *E*, *E′*, are left in open communication with the mercury in the leveling bulbs. Leveling bulb *L′* is now left slightly above the level of the chambers, and bulb *L* is rested about 10 cm. below the chambers. Serum is drawn into the chamber through tube *V*. The serum is thus drawn directly from the centrifuge tube to the chamber without exposure to air. The pinch-cock, *P*, at the top of the chamber is then closed, the glass bend, *V*, is removed, and the leveling bulb, *L*, is raised about 35 cm. above *L′* which is set just above the level of the chambers. To obtain a mercury seal at this point the chambers are revolved counterclockwise until the mercury in chamber *C* covers the opening of the capillary, *T*. The rubber tubing, *R*, between the clamp, *P*, and the capillary, *T*, is compressed to displace the serum in the capillary by mercury. The chambers should then be rotated clockwise, not to the upright position, but to one about 15° from the horizontal, to bring the serum into contact with the entire membrane (Fig. 3). If very small amounts of serum are used, the chambers may be rotated almost to the horizontal (in which case, of course, the filtrate will not remain in contact with the membrane). The filtrate displaces the mercury from the receiving chamber, *C′*, into its leveling bulb, *L′*. The effective filtering surface exceeds the 2.5 cm. aperture in the chamber because the membrane bulges considerably into the receiving chamber. The bulging is also advantageous in affording more adequate diffusion equilibrium. The apparatus is sufficiently compact to allow its use in the ordinary incubator or refrigerator, if desired.

After the required amount of ultrafiltrate has been formed, the chambers are rotated clockwise to the vertical position, the leveling bulbs are both brought to the middle of the stand and the mercury seals are displaced from the capillary tubes above the substrate and filtrate by compressing the rubber tubings, *R* and *R′*, above the capillaries. The substrate and ultrafiltrate can then be delivered into suitable containers, or directly into pipettes, if anaerobic handling is desired, by releasing the pinch-cocks. Pipettes calibrated to deliver between two marks (1) have been found to deliver substrate with protein concentration as high as 22 per cent quite as accurately as water.

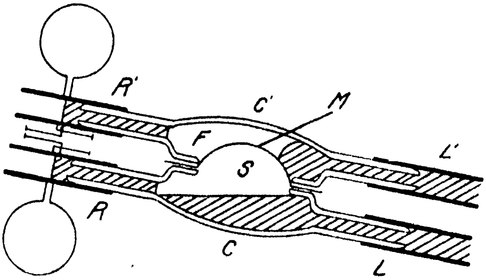

FIG. 3. Apparatus during filtration. The shaded areas represent mercury. The leveling bulb attached to *L* is placed at the top of the clamp stand, the other, *L′*, is set just above the level of the chambers. The membrane *M* is seen to bulge into the receiving chamber *C′*. Before removing the ultrafiltrate *F* and the substrate *S*, the chambers are rotated clockwise to the upright position and the mercury seals displaced from above them by compressing the rubber tubes *R* and *R′*.

The mercury left in the chambers is discarded to be cleaned before reusing. If the chambers are rinsed with cold water immediately after emptying, cleaning presents no difficulties. Alcohol and ether are used in drying the chambers, which are therefore practically sterile.

Observations

An effective pressure of approximately 35 cm. of mercury has been used throughout. Chambers of approximately 6 cc. capacity were used in most instances, although occasionally a larger one was used for the serum. Since the diameter of all the chambers is the same, they may be used interchangeably.

Reliability of Membranes—Commercially prepared cellophane is quite uniform in its properties, stronger than collodion, and, when washed free of glycerol, is as inert as filter paper. The glycerol can be completely removed by soaking in four or five changes of water, 1 minute or more being allowed for each soaking. The extracted material yields no ash. The membranes double in thickness in water within 3 hours (2) and may swell further on prolonged soaking. The swollen membranes hold back colloid particles only (2).

Membranes soaked in water for from 1 to 96 hours were tested for breaking strength while wet. With the chambers with the 2.5 cm. aperture, breakage usually occurred at 50 to 58 cm. of mercury with extremes at 45 and 62 cm. There is thus a considerable margin of safety when filtration is carried out at 35 cm. In a few instances when membranes were soaked more than a week before testing, breakage occurred at less than 35 cm. of mercury. This was not a constant occurrence, but it seems advisable to discard membranes which have soaked more than a week.

Repeated tests with heat and acetic acid for protein in ultrafiltrates of serum have proved negative. This was true even in an experiment in which filtration was greatly accelerated by carrying it on at 50°.

Speed of Filtration—Distilled water filtered at the rate of approximately 0.2 gm. per hour at 7°, and approximately twice as fast at 37°. Under the same conditions, filtrate was formed from serum at the approximate rates of 0.18 gm. per hour at 7°, 0.30 gm. per hour at 27°, and 0.37 gm. per hour at 37°. These rates were not materially altered by concentration of the substrate even when the filtrate was considerably in excess of the substrate. Varying the length of time during which the membranes were subjected to drying with air previous to use had no effect on the speed of filtration.

Tests for Loss of Carbon Dioxide and Destruction of Protein and Glucose during Filtration—Serum obtained with precautions against loss of CO_2 was subjected to anaerobic filtration at room temperature for 12 hours (see Table I, Experiments 1 and 2). The filtrate and substrate were then mixed without exposure to air to give reconstituted serum. In the first experiment the original serum CO_2 content was 65.4 volumes per cent, non-protein nitrogen 29 mg. per cent, and total protein 7.32 per cent; in the reconstituted serum the corresponding values were 65.6, 29, and 7.17. There occurred, then, no significant change in these constituents during ultrafiltration. In the second experiment in which CO_2 content alone was determined, agreement was equally good. In the remaining experiments, the CO_2 contents of serum, substrate, and filtrate were determined after variable periods. The volumes of substrate and filtrate were calculated from the concentrations of protein in the serum and substrate in Experiments 3 and 4 and were measured directly in the last two experiments (Table I). The values for "reconstituted serum" calculated from these data agree closely with those in the original serum.

TABLE I

Experiments to Test Apparatus for Loss of CO_2

Experiment No.	Time of filtration	Approximate volume of substrate	Approximate volume of ultrafiltrate	CO_2 content			
				Substrate	Ultrafiltrate	"Reconstituted serum"	Original serum
	hrs.	*cc.*	*cc.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
1	12	3	3			65.6	65.4
2	12	2	2			43.2	42.8
3	20	2	3	34.0	38.0	36.4	36.1
4	8	4	2	57.0	63.4	59.1	58.4
5	30	1.3	3.4	45.4	62.3	57.6	57.5
6	6	3.5	1.5	35.7	37.8	36.3	36.4

In three experiments, the pH was determined in serum directly, and in substrate and ultrafiltrate after ultrafiltration for 24 hours at room temperature. In one instance, the hydrogen ion concentrations in serum and filtrate were alike; in the others, that of serum lay between those of substrate and filtrate, indicating that no great change in hydrogen ions had occurred. The determinations were carried out with the glass electrode by Dr. L. F. Nims of the Department of Physiology.

Determinations of non-protein N in serum and substrate or filtrate have been made on several occasions. No significant changes were encountered, even when filtration was carried out at 37°. In one experiment determinations of nitrogen in the filtrates obtained from the same serum at 7° and at 50° were almost the same (22 and 18 mg. per cent respectively).

In a single experiment, glucose was found to be the same in the water of serum and in the ultrafiltrate obtained over 24 hours at 25°. In two experiments in which serum was drawn directly into sampling bulbs over mercury by the usual anaerobic technique, during 24 hours at 37° serum glucose was found to fall less than 10 per cent in one and not at all in the other, indicating that under the conditions of the ultrafiltration the breakdown of glucose must be very small.

Uniformity of Composition of Successive Portions of Ultrafiltrate and Effect of Temperature on Its Composition—In five experiments the ultrafiltrate from serum obtained with precautions against loss of CO_2 was sampled at two or three intervals, while the volume of the substrate decreased to as little as one-third the volume of the serum originally taken. Four similar experiments were conducted with serum containing sodium thiocyanate. Within the error of the methods of analysis (approximately ±1 per cent), no change in the concentration of either Cl of SCN in the ultrafiltrate was found as the volume of substrate diminished. This uniformity was secured only when the temperature was kept constant during the ultrafiltration. Much more SCN was filtrable at 37° than at 7°. Chloride showed a slight change in the same direction with change in temperature. Filtrable Ca decreases with rise of temperature (3).

DISCUSSION

Cellophane affords the usual advantages over collodion membranes; *i.e.*, greater strength, uniformity, and ability to dry completely without harm, thus avoiding dilution of serum. It is also particularly valuable because it does not adsorb protein (4). With collodion membranes the filtration of serum is much slower than that of water, the yield from serum decreasing, sharply during the 1st hour, and remaining reasonably constant thereafter (5), phenomena which are probably dependent on adsorption of protein by the membrane. With cellophane membranes in our apparatus, on the other hand, filtration of serum proceeds almost as rapidly as that of water. This is not surprising in the absence of adsorptive phenomena, since the osmotic pressure of the proteins is small in comparison with the filtration pressure of 35 cm. of mercury. For the same reason the failure of filtration to slow appreciably as the substrate becomes concentrated is to be expected. The increase in speed of filtration with rise of temperature can probably be referred to change in the viscosity of water, which is approximately halved when the temperature rises from 7° to 37° (6).

While the blood has been drawn with sterile technique in all experiments, it has been delivered into tubes and filtration chambers which are merely chemically clean. Nevertheless, analyses indicate no significant destruction of glucose or protein and no change in CO_2. No preservatives have been used.

The concentration of Cl in ultrafiltrate has been shown to remain essentially unchanged as the substrate becomes progressively inspissated, the concentration of Cl in substrate meanwhile falling progressively. This agrees with previous observations (5, 7). The concentrations of Ca, Mg, Na, and PO_4 in the ultrafiltrate are likewise practically independent of the relative volumes of filtrate and substrate (7, 8). The same is true of SCN which is present in much greater concentration in serum than in filtrate, being partly bound in some manner to the lipids of the serum (9). Concentrations of electrolytes in ultrafiltrate are, then, for practical purposes, independent of the relative volumes of substrate and filtrate. Comparisons of ultrafiltrate with the serum from which it is produced are therefore independent of the degree of concentration of the substrate.

SUMMARY

A method for the anaerobic ultrafiltration of serum is described.

No significant loss of carbon dioxide, glucose, or protein occurs during the process.

For practical purposes, the concentration of electrolytes in the ultrafiltrate is independent of the relative volume of substrate and filtrate.

Change in temperature, however, affects the concentrations of these substances in ultrafiltrate.

BIBLIOGRAPHY

1. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 17 (1932).
2. McBain, J. W., and Kistler, S. S., *J. Gen. Physiol.*, **12**, 187 (1928).
3. Lewis, R., Hald, P. M., and Robbins, C. L., unpublished experiments.
4. Dow, P., *J. Gen. Physiol.*, **19**, 907 (1936).
5. Augsberger, A., *Biochem. Z.*, **196**, 276 (1928).
6. Washburn, E. W., International critical tables of numerical data, physics, chemistry and technology, New York, **5**, 10 (1929).
7. Greene, C. H., and Power, M. H., *J. Biol. Chem.*, **91**, 183 (1931).
8. Hirth, and Tschimber, C., *Compt. rend. Soc. biol.*, **91**, 592 (1924).
9. Rosenbaum, J. D., and Lavietes, P. H., unpublished experiments.

STUDIES ON KETOSIS

XII. THE EFFECT OF CHOLINE ON THE KETONURIA OF FASTING RATS FOLLOWING A HIGH FAT DIET*

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Although there is considerable evidence that choline is able to prevent the accumulation of fat in the liver as a result of a high fat diet (1), the mechanism by which this is possible is still not understood. Thus, Gaddum (2) states that, "The effect [of choline] is apparently not due to an action on the absorption or excretion of fat, and the most probable explanation is that choline stimulates the liver to oxidise both neutral fats and cholesterol esters." If such is the explanation for the action of choline, then it should markedly increase the ketonuria in fasting rats which have fatty livers; moreover, it should cause the oxidation of ingested fat with the result that no ketonuria should be produced when animals, previously on a high fat diet and choline, are subsequently fasted.

In a recent report (3) we have shown that a ketonuria of considerable magnitude ensues in rats during fasting following a high fat diet which causes the deposition of considerable amounts of fat in the liver. However, the degree of the ketonuria was not directly proportional to the height of the liver fat. On the other hand, there was practically no acetonuria in rats previously fed our stock diet low in fat. In experiments with a variety of fats

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* This work was assisted by a research grant from the Rockefeller Foundation.

Some of these data are included in a thesis submitted by David B. Tyler to the Department of Physiology in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

the level of ketonuria was invariably higher in female rats than in the males. This was apparently not to be ascribed to higher values in liver fat at the start of the fast but rather to a more labile liver fat in the female rats.

In the present investigation, a study has been made on the excretion of ketone bodies in the urine of animals previously on a high fat diet with or without choline, and the effect of the administration of choline to such animals during the period of fasting has also been investigated.

Procedure

The diets were similar to those employed in our earlier work (3) except that choline hydrochloride (plus enough $NaHCO_3$ to neutralize the HCl) was mixed with the diet. The choline added was 10 gm. per kilo of food in Series I, and 40 gm. per kilo of food in Series II. On the basis of the average food intake of 4 gm. per day, this gave 38 mg. per rat per day in the first case (Series I), and with an average of 2.5 gm. of food eaten in the second group (Series II) there were 96 mg. of choline taken in.

Choline hydrochloride (neutralized with $NaHCO_3$) was given by stomach tube in doses of 50 to 225 mg. per day in two divided doses during the fasting period in some tests. The glycogen, water, and fat of the liver were determined in a control group without fasting; while liver lipids and water were ascertained in animals following periods of inanition of 3, 4, or 5 days during which the ketonuria was followed. The analyses on the liver and urine were similar to those employed in our earlier work (3).

Results

The analyses of the livers of unfasted rats previously receiving the high fat diet with or without choline, for water, glycogen, and fat, are summarized in Table I. Table II records the water and lipid content of the livers of animals on similar diets after various periods of fasting during which the ketonuria was studied.

The administration of choline during the period that the high fat diet was being ingested prevents the accumulation of any appreciable amount of fat in the liver. The average liver lipid of the male rats on choline was 4.27 per cent compared with a control value of 32.35 per cent. The levels with the females were 3.67

and 2.85 per cent respectively for the low and high choline diets, compared with a control percentage of 28.43.

On the other hand, fasting is followed by a considerable increase in liver fat in the case of animals previously on a choline-high fat diet. Average values of liver fats of males increased to 7.83 per cent, while those of females rose to 9.23, 7.48, and 8.80 per cent respectively after 3 to 5 days of fasting.

TABLE I

Water, Glycogen, and Lipid in Livers of Unfasted Rats Previously Receiving High Butter Fat Diet for 14 Days with and without Choline

Sex	Previous diet	No. of experiments	Body weight	Liver	Water	Glycogen	Lipid
			gm.	*per cent body weight*	*per cent*	*per cent*	*per cent*
Males	Butter fat*	15	194	4.86	50.0	3.05	32.35
	Choline I	8	184	3.17	69.4	5.86	4.27
						9.18	**28.08**
Females	Butter fat*	20	144	5.34	48.6	1.51	35.26
	" " †	8	151	4.79	53.6	2.13	28.43
	Choline I	20	150	3.57	70.0	5.45	3.67
						14.42	**44.30**
	" II	5	132	3.12	69.8	1.92	2.85
							45.50

The figures in bold-faced type represent the ratio of the mean difference to the probable error of mean difference of the choline tests (directly above) when compared with the corresponding sex group which received only the butter fat diet.

* These results are the averages from the experiments of Deuel, Hallman, and Murray (3).

† Values for the control rats carried out in this study.

The effect of choline on the liver lipid when administered during the fasting period is given in Table III.

The administration of choline to rats previously on the butter fat diet increased the rate of breakdown of the liver fat. Thus, the average for the liver lipid of nine male controls killed without fasting was 20.40 per cent, while that of the litter mate controls which were fasted 4 days was 18.88 per cent. On the other hand, when choline was administered during the fast, the average level of twelve animals had fallen to 12.98 per cent after 4 days (or 11.35 per cent if one omits the consideration of one abnormally high member of the group in which the liver lipid was 31.37 per cent).

TABLE II

Water and Lipid in Livers of Rats Fasted for Various Periods after Receiving High Butter Fat Diet for 14 Days with or without Choline (No Choline during Fast)

Sex	Previous diet	No. of experiments	Fast	Body weight		Liver	Water	Lipid	M.D.:P.E.M.D.*
				Start	End				
			days	*gm.*	*gm.*	*per cent body weight*	*per cent*	*per cent*	
Males	Butter fat†	10	3	187	177	4.27	52.2	30.59	
						−0.59	**+2.2**	**−1.76**	
	Choline I	7	4	200	168	3.02	69.2	7.83	7.32
						−0.15	**−0.1**	**+3.56**	
Females	Butter fat†	9	3	157	133	5.19	51.9	30.38	
						−0.15	**+3.3**	**−4.88**	
	Choline I	5	3	146	121	3.18	67.1	9.23	2.71
						−0.39	**−2.9**	**+5.56**	
	" II	5	3	135	118	3.12	69.4	5.66	2.89
						0.00	**−0.4**	**+3.71**	
	" I	5	4	149	121	3.57	69.7	7.48	4.17
						0.00	**−0.3**	**+3.81**	
	Butter fat‡	10	5	148	124	4.06	59.0	22.58	
						−1.00	**+7.8**	**−9.27**	
	Choline I	10	5	172	146	2.76	68.7	8.80	4.98
						−0.81	**−1.3**	**+5.13**	

The figures in bold-faced type represent the change from the averages recorded in Table I of unfasted animals.

* Comparison with experiments on unfasted rats (Table I).

† Experiments of Deuel, Hallman, and Murray (3).

‡ Averages of two groups of female rats (five in each group) fasted 5 days. Experiments on one group were carried out simultaneously with the present tests; the other group is from Deuel, Hallman, and Murray (3). The changes are calculated from the averages of two groups in Table I of unfasted female rats previously on butter fat.

In five cases after the administration of choline during the fast the level of liver fat had fallen below 10 per cent, the individual levels being 4.26, 5.23, 5.43, 5.02, and 9.11 per cent. Although the results on female rats are fewer in number, it is also evident here that there is a greater decrease in liver fat in the animals which received choline.

When the liver fat has been maintained at minimum values by the inclusion of choline in the diet, its level is kept at a minimum value during fasting if the administration of choline is continued. Thus, the mean value for liver lipid of female rats under such an experimental régime was 3.78 per cent compared with a level of 22.38 or 22.77 per cent for rats previously on a butter fat diet and fasted 5 days, or with values of 8.80 per cent for rats on the Choline I diet but without choline during the fasting period.

TABLE III

Water and Lipids in Livers of Rats Fasted for Various Periods but Receiving Choline after Diets High in Butter Fat with or without Choline

Sex	Previous diet	No. of experiments	Fast	Choline per day during fast	Body weight		Liver	Water	Lipid	M.D.:P.E.M.D.*
					Start	End				
			days	*mg.*	*gm.*	*gm.*	*per cent body weight*	*per cent*	*per cent*	
Males	Butter fat	5	5	0	231	204	3.15 **−0.46**	61.6 **+3.0**	18.88 **−1.52**	
	" "	12	5	225	248	218	2.89 **−0.72**	65.7† **+7.1**	12.98† **−7.42**	3.02
Females	" "‡	10	5	0	148	124	4.06 **−1.00**	59.0 **+7.8**	22.58 **−9.27**	
	" "	5	5	50−200	154	131	3.34 **−1.72**	64.4 **+13.2**	16.50 **−15.16**	
	Choline I	10	4	100	152	123	3.37 **−0.20**	72.3 **+2.3**	3.78 **+0.09**	

The figures in bold-faced type represent the change from the averages of values of unfasted animals on which experiments were carried on simultaneously.

* Comparison with control group fasted 5 days without choline.

† Except for one abnormal result, the liver water for eleven experiments averaged 66.7 per cent and the liver lipid 11.35 per cent.

‡ Averages of two groups of female rats (five in each group) fasted 5 days. Experiments on one were carried out simultaneously with the present tests; the other group is from Deuel, Hallman, and Murray (3). The changes are calculated from the averages of two groups in Table I of unfasted female rats previously on butter fat.

During the periods of fasting after the high butter fat diets, studies on ketonuria were made. At the conclusion of the ketonuria studies, the animals were sacrificed and the analyses on the liver carried out. The records of the ketonuria studies are summarized in Tables IV and V.

TABLE IV

Acetone Body and Nitrogen Excretion in Urine of Fasting Rats Previously Receiving High Butter Fat Diets with and without Choline (No Choline during Fast)

Sex	Previous diet	Body weight	Acetone bodies, gm. per sq. m.					Urine N, gm. per sq. m.				
			1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day
		gm.										
Males	Butter fat*	172	0.06 (14)	1.80 (15)	1.88 (15)	1.46 (5)		2 55	2.87	3.01	3 11	2 79
	Choline I	198	0.03 (7)	0.71 (7) **3.46†**	2.18 (7) **0.84†**	1.93 (7) **1.46†**		2.15	2.61	2.72	2.46	
Females	Butter fat‡	152	0.69 (18)	3.09 (19)	3.15 (19)	2.32 (10)	1.61 (10)	2.51	2 97	2 62	2.70	2.52
	Choline I	160	0.01 (20) **5.11†** **3.41§**	1.56 (20) **5.49†** **3.79§**	3.48 (20) **1.33†** **2.32§**	2.81 (15) **1.57†**	1.96 (10) **1.34†**	2.21	2.80	2.59	2.34	2.07
	Choline II	135	0.01 **5.11†**	0.27 **12.92†**	1.16 **5.69†**			2.14	3.02	2.88		

The figures in parentheses represent the number of experiments included in the averages.

* Results of Deuel, Hallman, and Murray (3).

† M.D.: P.E.M.D. compared with butter fat controls.

‡ Combined results of tests carried on simultaneously and of results of Deuel, Hallman, and Murray (3).

§ Comparison of M.D.: P.E.M.D. between males and females previously on Choline I diet.

The extent of ketonuria in fasted animals which had previously received the high fat diet with choline is significantly lower than that of the control rats on a similar diet without choline for the first 2 fast days. Thereafter there was a higher level (but not statistically significant) from the 3rd to the 5th days of the fast on the rats previously on the low choline diet than was noted in

Table V

Acetone Body and Nitrogen Excretion in Urine of Fasting Rats Receiving Choline during Fast after Previously Receiving High Butter Fat Diets with or without Choline

Sex	Previous diet	Body weight	Choline fed during fast	Acetone bodies, gm. per sq. m.					Urine N, gm. per sq. m.				
				1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day
		gm.	*mg.*										
Males	Butter fat*	188	0	0.04(20)	1.81(20)	2.03(20)	1.63(10)	1.02(9)	2.44	2.79	2.92	3.11	2.79
	" "	247	200	0.05(12)	1.83(12)	2.48(12)	0.72(12)	0.49(12)	2.16	2.70	2.84		
							4.26†	**2.37**†					
Females	" " *	152	0	0.69(18)	3.09(19)	3.15(20)	2.32(10)	1.61(10)	2.51	2.97	2.62	2.70	2.52
	" "	154	50-200	0.13(5)	3.14(4)	3.67(5)	2.22(5)	1.71(5)	2.75	3.18	2.81	2.83	2.26
	Choline I	152	100	0.00	0.56(10)	1.16(10)	1.12(10)		2.19	3.00	2.82	2.78	
					5.82‡	**9.28**‡	**5.83**‡						

The figures in parentheses represent the number of experiments of which the preceding figure is the average.

* Experiments of Deuel, Hallman, and Murray (3) and results of control tests of present experiments.

† M.D.: P. E. M. D. when compared with the level of butter fat controls without choline.

‡ M.D.: P. E. M. D. when compared with animals previously on Choline I (Table IV) but with no choline during fasting.

the control rats. As in rats fed with the high fat diets in the absence of choline (3), the sex difference in ketonuria is also evident in the rats fed on the Choline I diet. Significantly higher values are obtained with the females from the 2nd to 4th days, the duration of the tests with the males.

The results in Table IV indicate that the choline from the previous diet may be stored for several days. In contrast with the relatively high ketonuria which is present on the 1st day with the control females (0.69 gm. per sq. m.), the results are blank for the animals previously on the choline diet (0.01 gm. per sq. m.). The effect of the previous choline feeding persists for the first 2 days in both the males and females previously on the low choline diet (Choline I). It is more pronounced on the first 2 days and persists for the 3rd fast day in the rats previously administered the high choline diet (Choline II).

The administration of large doses of choline to male or female rats previously on the high butter fat diet during the fasting period does not significantly alter the course of the ketonuria on the first 3 days. The results on males (Table V), however, definitely demonstrate a significant lowering of the level of acetone body excretion during the 4th and 5th days over that of the control group. Such variations could not be noted in the small group of females.

When choline is administered in a dose of 100 mg. per day during fasting to female rats previously on the high fat diet with choline (Choline I), not only is the migration of fat to the liver prevented during the fasting period (Table III), but also the ketonuria is kept at a minimum value which is significantly lower than that of animals on a similar diet previous to the fasting period but which received no additional choline during the fast.

In all cases there was a drop in weight of less than 10 per cent during the high fat diet. Food consumption averaged somewhat more than 4 gm. per 100 gm. of body weight per day with the rats receiving the butter fat diet, while there was a mean of about 3.5 gm. per 100 gm. per day for the tests with the Choline I groups. In the Choline II tests, only 2.5 gm. of food were consumed per day per 100 gm. of rat.

DISCUSSION

Although the administration of choline with a high butter fat diet is sufficient to prevent the accumulation of an appreciable amount of fat in the liver, it only temporarily prevents the development of a ketonuria in a subsequent fast period if the administration of the base is not continued during the period of inanition. In fact the subsequent ketonuria reaches a somewhat higher magnitude than that of the fasting controls which had previously received a similar diet without choline. Such a phenomenon is accompanied by a concomitant rapid infiltration of fat into the liver. Whereas the liver lipid of unfasted rats on a choline-containing butter fat diet was only 4.27 and 3.67 per cent in male and female rats respectively, this had increased to a mean of 7.83 per cent in male rats after a 4 day fast and to levels of 9.23, 7.48, and 8.80 per cent of lipid in female rats fasted 3, 4, and 5 days respectively.

The average liver lipid in unfasted rats previously on our stock diet (3) was found to be 3.76 and 3.79 per cent for male and female rats respectively, while this was changed to means of 3.95 and 3.61 per cent respectively after 3 days of inanition and to 4.31 and 4.07 per cent after 5 days of fasting. The acetone body excretion of rats previously fed on our stock diet was practically negligible. The average elimination of ketone bodies in the male rats amounted to 0.04, 0.03, 0.05, 0.17, and 0.30 gm. per sq. m. over the 5 day period respectively, while the values in the females were 0.04, 0.01, 0.10, 0.13, and 0.35 gm. per day respectively. Therefore it becomes apparent that the infiltration of fat in the liver and the extent of the ketonuria in the animals previously fed choline cannot be traced solely to the fast period but must be related to the preceding diet.

We are forced to the conclusion that in spite of the fact that choline prevents the deposition of labile fat in the liver, it cannot inhibit the storage of such a readily metabolizable lipid in the tissues. Unless such material were so stored in our choline-fed rats, why should an infiltration of fat occur during fasting and why should a ketonuria ensue during the subsequent inanition out of all proportion to that which occurs in animals previously fed the stock diet which is low in fat? In spite of the fact that fasted animals on the latter diet (stock diet) have large amounts of available fat in the tissues, neither does such an infiltration occur nor does such an acetonuria ensue as in the choline-fed rats.

The transfer of the tissue fat to the liver must be an aid to its oxidation. When this transfer is prevented during the fast period by a continued choline intake, the ketonuria is significantly lowered although not completely prevented during the fast.

Choline does not increase the extent of oxidation of fat but rather decreases it if the extent of ketone body elimination is to be considered an index of the extent of fat breakdown. Thus, when large amounts of choline were administered daily to fasting rats with high levels of liver fat (previously on a butter fat diet without choline), no alteration in ketonuria could be noted during the first 3 fast days in males nor during 5 days in females. Moreover, the level of ketonuria was significantly lower in the males so treated during the 4th and 5th days than in rats receiving no choline. Under such conditions it would seem that the lower level of liver fat ultimately found in the rats fed choline during the fast must be explained by the retransfer of such liver fat to the tissues rather than to its oxidation or to the prevention of the migration of additional fat to the liver from the tissues during the fasting period. Also in animals on the Choline I diet, the administration of choline during the entire fast not only did not increase the level of ketonuria but significantly lowered it.

The lower level of acetonuria during the first 2 fast days in rats previously fed the Choline I diet (as compared with the controls receiving butter fat) might be caused either by the prolonged sparing effect of carbohydrate in the former case or to the retention of sufficient choline to prevent the transfer of the labile tissue fat to the liver. The liver glycogen of the unfasted male and female rats previously on the Choline I diet was 5.86 and 5.45 per cent respectively, compared with values of 3.05 and 1.51 per cent respectively in rats previously on a similar high butter fat diet without choline. This is the highest level of liver glycogen we have noted in adult unfasted female rats receiving a variety of high fat diets, or even in rats on the stock diet (3), while in only one case (cod liver oil diet) has such a high mean for liver glycogen been equaled in the males. In most high fat diets liver glycogen in the males approximated 3 per cent and that of the females 2 per cent. Whether such high glycogen values are due to a specific effect of the choline on carbohydrate storage in the liver is the subject of active investigation at present in our laboratory. Another possible explanation of the high level is that it is not a specific effect of choline but results from the high glucose diet (48 per cent) when the glycogen is not crowded out of the liver by an excessive fat deposition.

On the other hand, other evidence points rather to the fact that the lowered ketonuria during the early fast days is to be attributed to a retention of the effect of choline for several days during the fast period. The most cogent evidence is found in the experiments in which the high dose of choline in the food was employed (Choline II). In these experiments the level of liver glycogen of the unfasted female rats is only 1.92 per cent. This is presumably to be traced to the lowered food consumption of this group because the diet was distasteful with the larger choline content. In spite of the initial lower level of glycogen, however, the inhibitory effect on ketonuria is more prolonged and was still noted on the 3rd fast day, after which the experiments were discontinued. Another fact which indicates that the initial level of liver glycogen is not a factor in the lower level of ketonuria is that in the males which previously received the cod liver oil diet (3) the ketonuria not only was not lowered from the level of the rats receiving butter fat but was actually greater on both the 1st and 2nd days of the fast.

SUMMARY

The administration of choline to rats on a high butter fat diet prevents the accumulation of fat in the liver. However, during a subsequent fast period a considerable infiltration of fat into the liver occurs.

The level of ketonuria is lower during the first 2 fast days and higher during the following 3 fast days in rats previously on the choline-butter fat diet than on the butter fat controls. Administration of choline during the fast to rats which previously received no choline and had high liver fat did not alter ketonuria during the first 3 days but lowered it significantly in the males on the 4th and 5th days. The liver fat, likewise, was lowered more rapidly in the animals which received choline during the fast. The administration of choline during the period of inanition to rats previously receiving the choline-butter fat diet signficantly lowered the extent of ketone body production, and also prevented the infiltration of fat into the liver.

On the basis of these results it is concluded that choline does not increase the rate of fat oxidation (if ketonuria during fasting is an evidence of such oxidation). Choline prevents the deposition of labile fat in the liver but cannot prevent such deposition in the

tissues. On subsequent fasting such fat is transferred rapidly to the liver. Such data would seem to open the question as to whether the conversion of fat to lecithin is a preliminary step in its oxidation. It would seem that the accumulation of lecithin might tend to prevent the oxidation of fat if the choline in that molecule behaves in a similar manner to free choline.

BIBLIOGRAPHY

1. Best, C. H. Hershey, J. M., and Huntsman, M. E., *J. Physiol.*, **75**, 56 (1932).
2. Gaddum, J. H., in Luck, J. M., Annual review of biochemistry, Stanford University, **4**, 316 (1935).
3. Deuel, H. J., Jr., Hallman, L. F., and Murray, S., *J. Biol. Chem.*, **119**, 257 (1937).

STUDIES IN AMINO ACID METABOLISM

III. THE FATE OF *dl*-LEUCINE, *dl*-NORLEUCINE, AND *dl*-ISOLEUCINE IN THE NORMAL ANIMAL*

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Previous communications from this laboratory have dealt with the fate of various glycogenic amino acids (1, 2) when fed to the normal animal. The data show a superiority in the metabolism of the natural isomer when compared to the racemic mixture. This difference has been demonstrated with alanine, glutamic acid, and aspartic acid.

In the present study, similar experiments have been made with leucine, isoleucine, and norleucine. The information about the fate of these compounds is quite fragmentary. Simon (3) could not show any glycogen after feeding leucine to rabbits which had previously been rendered glycogen-free by strychnine. Wilson and Lewis (4) were unable to find any rise in liver or body glycogen of normal rats after feeding this amino acid. Halsey (5) failed to demonstrate glucose formation in a phlorhizinized dog after feeding leucine. On the other hand, Embden, Solomon, and Schmidt (6) reported a great increase in acetone bodies when leucine was added to perfusing liquid passed through a surviving liver. The natural isomer was especially effective in this regard, while its optical isomer was less effective (Embden (7)). When leucine was fed to a diabetic patient, Baer and Blum (8) also found an increased output of β-hydroxybutyric acid. Dakin (9) reported that neither sugar nor acetone bodies were present in the urine after isoleucine was given to a phlorhizinized dog, although Wirth

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(10) reported that sometimes acetone bodies were found when dogs' livers were perfused with isoleucine. Of the possible pathway of metabolism nothing is known. Czarnetzky and Schmidt (11) have presented data which show that norleucine is a naturally occurring amino acid. Greenwald (12), using the phlorhizin technique, has demonstrated "extra sugar" after feeding the racemic as well as the *d* and *l* forms of norleucine.

EXPERIMENTAL

Following the procedure used previously, we have made a study of the glycogenic properties and ketolytic or ketogenic behavior of these three leucines, namely, *dl*-leucine, *dl*-norleucine, *dl*-isoleucine fed as the sodium salts.

Female rats ranging from 100 to 160 gm. in weight were used for the glycogen studies. After a preliminary 48 hour fast the animals were fed hourly an amount greater than could be absorbed during this period, so that maximum absorption was obtained at all times. At the end of 2, 4, 6, 8, 10, and 12 hours a group of animals was sacrificed, with amytal as an anesthetic, the livers were removed, and glycogen determinations were carried out according to the method of Good, Kramer, and Somogyi (13). At the same time the gastrointestinal tract was removed, washed out, and after treatment with trichloroacetic acid the washings were filtered. Analyses were made on the washings for amino nitrogen according to the Van Slyke procedure. The report of the experiments on absorption of various amino acids will be the subject of a later communication from this laboratory. As reported earlier (2), filter paper was placed in the cages during the fast period and the animals were allowed to eat this *ad libitum.*

Since we were dealing with amino acids, at least one of which is believed to be ketogenic, we felt a comparison of the acetone body output after the amino acid alone as well as the excretion when acetoacetic acid and the amino acid combined were fed might give us a better picture of the metabolism. Urine collections were made every 24 hours, as described previously (14). Analyses for total acetone bodies were carried out according to the Van Slyke technique and for total nitrogen by the Kjeldahl method. Male and female rats of similar age to those used in the glycogen tests were employed in the studies on ketonuria.

TABLE I

Glycogen Content of Livers from Female Rats Fasted 48 Hours and Receiving Sodium Salts of Various Amino Acids for Varying Time Intervals

Maximum absorption occurred at all times. The results are expressed in per cent.

Amino acid fed	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
dl-Leucine	0.07 (5)	0.09 (4)	0.10 (5)	0.05 (5)	0.08 (5)	0.11 (5)
dl-Isoleucine	0.08 (5)	0.08 (5)	0.10 (6)	0.19 (8)	0.64 (5)	0.53 (5)
					8.76	**7.01**
dl-Norleucine	0.08 (5)	0.07 (5)	0.20 (5)	0.77 (4)	0.99 (5)	1.29 (5)
				5.40	**11.50**	**12.40**

Control, fourteen animals = 0.19 per cent.

The figures in parentheses refer to the number of animals used.

The figures in bold-faced type represent the ratio of the mean difference to the probable error of the mean difference as compared to the controls.

TABLE II

Acetone Body Excretion (Gm. per Sq. M.) from Female Rats Receiving 15.00 Gm. of Acetoacetic Acid per Sq. M. per Day (Calculated As Acetone) Along with dl-Norleucine, dl-Leucine, or dl-Isoleucine Fed with One-Fourth the Theoretical Amount of Sodium Bicarbonate

Material fed	Amount fed, per sq. m.	Acetone body output				
		1st day	2nd day	3rd day	4th day	Average for 4 days
dl-Norleucine	11.60 gm. or 5.15 gm. as acetone + acetoacetic acid 15.00 gm.	3.04 (8)	2.27 (8)	3.69 (8)		3.00 (24)
"	22.60 gm. or 10.00 gm. as acetone + acetoacetic acid 15.00 gm.	3.54 (3)	0.85 (2)	1.25 (1)	2.40 (2)	2.29 (8)
dl-Leucine	22.60 gm. or 10.00 gm. as acetone + acetoacetic acid 15.00 gm.	7.82 (4)	7.57 (4)	11.90 (4)	13.36 (4)	10.14 (16)
dl-Isoleucine	22.60 gm. or 10.00 gm. as acetone + acetoacetic acid 15.00 gm.	6.13 (4)	6.40 (4)	9.21 (3)	13.05 (3)	8.34 (14)
Acetoacetic acid + saline	15.00 gm. as acetone + saline	4.87 (12)	6.50 (12)	7.44 (9)	8.19 (4)	6.39 (37)

The figures in parentheses indicate number of animals used.

Results

In a control group of fourteen female rats the values for liver glycogen ranged from 0.06 to 0.50 per cent, with an average of 0.19 per cent. In Table I are given the results on liver glycogen for animals in which maximum absorption was allowed for 2, 4, 6, 8, 10, and 12 hours after the sodium salts of *dl*-leucine, *dl*-isoleucine, and *dl*-norleucine were fed. None of the values after

TABLE III

Acetone Body Excretion (Gm. per Sq. M.) from Male Rats Receiving Only Sodium Salt of dl-Leucine or dl-Norleucine

Material fed	Amount fed per day	1st day	Per cent β-hydroxybutyric acid	2nd day	Per cent β-hydroxybutyric acid	3rd day	Per cent β-hydroxybutyric acid	4th day	Per cent β-hydroxybutyric acid
	gm. per sq. m.								
dl-Leucine	10.00 as acetone	3.81	66.6	4.33	67.8	1.65	63.0	2.87	57.5
	22.60 " leucine	(5)		(5)		(4)		(2)	
dl-Norleucine	10.00 " acetone	0.31		0.09		0.10		0.14	
	22.60 " norleucine	(4)		(4)		(4)		(4)	
Sodium bicarbonate	Equivalent to amount of sodium contained in leucines	1.02		0.97		1.27	81.0	0.46	75.5
		(4)		(4)		(4)		(4)	

The figures in parentheses refer to the number of animals used.

TABLE IV

Total Acetone Body Excretion (Gm. per Sq. M.) of Female Rats Receiving 22.60 Gm. per Sq. M. of Body Surface per Day of Various Leucines (As Sodium Salts)*

The control animals received an equivalent amount of sodium acetoacetate.

Material fed	1st day	2nd day	3rd day	4th day
dl-Leucine	0.95 (7)	3.54 (7)	3.63 (7)	4.49 (7)
dl-Isoleucine	0.62 (7)	0.79 (2)	1.89 (7)	1.33 (2)
dl-Norleucine	0.21 (7)	0.19 (7)	0.20 (7)	0.22 (7)
Sodium acetoacetate	1.35 (5)	5.15 (5)	7.00 (5)	9.00 (5)

The figures in parentheses refer to the number of animals used.

* This corresponds to 10 gm. calculated as acetone.

the *dl*-leucine shows any glycogen whatsoever. In fact, all of these were even lower than was the average control. Significant glycogen deposition was found after the *dl*-isoleucine for 10 and 12 hours, with values of 0.64 and 0.53 per cent respectively. After *dl*-norleucine was fed for 8, 10, and 12 hours, the livers of the rats showed a glycogen content of 0.77, 0.99, and 1.29 per cent respectively. Statistical treatment of these data is given in Table I.

The acetone body output after the three leucines does not present such a clear picture in all cases. Apparently the ketolytic activity follows the same order as the glycogen studies. When fed without acetoacetic acid, *dl*-leucine was shown to be definitely ketogenic. These results are given in Tables II, III, and IV.

DISCUSSION

Glucose is definitely an end-product of the metabolism of *dl*-norleucine. Although the absorption of this acid is quite slow (15), appreciable glycogen deposition occurred in the 8, 10, and 12 hour periods. These values of 0.77, 0.99, and 1.29 per cent are significant when compared to the control level of glycogen or that of the animals receiving *dl*-leucine. That *dl*-norleucine is a glucose former is further indicated in the experiments in which it is demonstrated that it exerts a marked ketolytic effect when fed either in a dose of 11.60 gm. per sq. m. or at a level approximately twice as great. It seems probable that deamination occurs, followed by subsequent destruction of the terminal carboxyl group and oxidation of the α-carbon atom to an acid group. This would leave *n*-valeric acid, which has been shown to be a glycogen former (16). Presumably β oxidation occurs, with formation of propionic acid which is converted to sugar.

dl-Leucine is convertible to the acetone bodies, as demonstrated by the increased acetone body production which occurs when the sodium salt of this acid is fed to rats having a ketonuria induced by the administration of sodium acetoacetate, as well as by the marked acetonuria which is induced when this amino acid is fed alone to fasting rats. As is shown in Table III, the distribution between the fractions is approximately 65 per cent for β-hydroxybutyric acid and 35 per cent for acetone and acetoacetic acid. The β-hydroxybutyric acid fraction is slightly lower than that obtained after feeding sodium acetoacetate (17) or ethyl acetoacetate

(18), and markedly decreased from the value obtained after the administration of the ethyl esters of the even chain fatty acids from butyric to stearic acid (18). In the latter case the β-hydroxybutyric acid fraction varied from 77.7 to 84.3 per cent. The failure to form glycogen indicates that leucine cannot be changed to glucose. The glycogen levels actually are below those of the controls. We have noted earlier, when feeding non-sugar-forming fatty acids, that quite often the liver glycogen level is depressed below that of the controls. We do not know whether this lowering is significant or due to biological variation; however, it seems to occur quite consistently.

Probably the most interesting results were obtained with *dl*-isoleucine (Table IV). This compound in the 10 and 12 hour periods shows significant liver glycogen deposition. Furthermore, not one of the controls is as high as the average value of these two periods. When the mean difference is divided by the probable error of the mean difference, values of 8.76 and 7.01 are given. Statistically if this ratio is greater than 3, significance can be given to the results.

On the other hand, the values after feeding the sodium salt of this acid alone, as reported in Table IV, and after feeding it along with acetoacetic acid, as shown in Table II, indicate ketogenic properties under these conditions. The values are less than after leucine feeding, but show definite acetone body production.

The most logical conclusion as to the pathway of metabolism seems to be that either deethylation or demethylation may occur. That the former may take place was first suggested by Wirth (10) who demonstrated that acetone bodies may or may not be formed in livers perfused with an isoleucine solution. The latter procedure has long been postulated to explain the formation of acetone bodies from leucine. The first would leave as an end-product a 3-carbon acid, possibly pyruvic acid, while a 4-carbon acid, probably acetoacetic acid, should be formed in the second instance.

SUMMARY

1. *dl*-Leucine when fed to the normal animal does not give rise to glycogen but does contribute to acetone body formation.
2. *dl*-Norleucine gives rise to an appreciable amount of glycogen and exhibits marked ketolytic properties.

3. *dl*-Isoleucine forms a small amount of glycogen, and under certain conditions may also give rise to acetone bodies.

BIBLIOGRAPHY

1. Butts, J. S., Dunn, M. S., and Hallman, L. F., *J. Biol. Chem.*, **112,** 263 (1935-36).
2. Butts, J. S., Blunden, H., and Dunn, M. S., *J. Biol. Chem.*, **119,** 247 (1937).
3. Simon, O., *Z. physiol. Chem.*, **35,** 315 (1902).
4. Wilson, R. H., and Lewis, H. B., *J. Biol. Chem.*, **85,** 559 (1929-30).
5. Halsey, J. T., *Am. J. Physiol.*, **10,** 229 (1903-04).
6. Embden, G., Solomon, H., and Schmidt, F., *Beitr. chem. Physiol. u. Path.*, **8,** 129 (1906).
7. Embden, G., *Beitr. chem. Physiol. u. Path.*, **11,** 348 (1908).
8. Baer, J., and Blum, L., *Arch. exp. Path. u. Pharmakol.*, **55,** 89 (1906).
9. Dakin, H. D., Oxidations and reductions in the animal body, Monographs on biochemistry, London, 2nd edition, 75 (1922).
10. Wirth, J., *Biochem. Z.*, **27,** 20 (1910).
11. Czarnetzky, E. J., and Schmidt, C. L. A., *J. Biol. Chem.*, **97,** 333 (1932).
12. Greenwald, I., *J. Biol. Chem.*, **25,** 81 (1916).
13. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100,** 485 (1933).
14. Butts, J. S., and Deuel, H. J., Jr., *J. Biol. Chem.*, **100,** 415 (1933).
15. Butts, J. S., Blunden, H. D., and Dunn, M. S., unpublished results.
16. Deuel, H. J., Jr., Butts, J. S., Hallman, L. F., and Cutler, C. H., *J. Biol. Chem.*, **112,** 15 (1935-36).
17. Butts, J. S., Cutler, C. H., Hallman, L. F., and Deuel, H. J., Jr., *J. Biol. Chem.*, **109,** 597 (1935).
18. Deuel, H. J., Jr., Hallman, L. F., Butts, J. S., and Murray, S., *J. Biol. Chem.*, **116,** 621 (1936).

RELATIONS OF THIOUREA, CYSTEINE, AND THE CORRESPONDING DISULFIDES*

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In his studies on the catalysis of the oxidation of cysteine and glutathione by hydrogen peroxide Pirie (1933) established that thiourea and a number of its derivatives, notable among which is ergothioneine, catalyze this reaction in a manner similar to the catalysis by copper ions. In investigating the catalytic mechanism he showed that dithioformamidine, the disulfide corresponding to thiourea, which is readily formed by hydrogen peroxide oxidation of the latter, rapidly reacts with 2 molecules of cysteine to form cystine. Pirie concluded that the mechanism is[1]

$$2\text{UH}{=}\text{S} + \text{H}_2\text{O}_2 \rightarrow \text{U}{-}\text{S}{-}\text{S}{-}\text{U} + 2\text{H}_2\text{O} \qquad (1)$$

$$2\text{Cy}{-}\text{SH} + \text{U}{-}\text{S}{-}\text{S}{-}\text{U} \rightarrow \text{Cy}{-}\text{S}{-}\text{S}{-}\text{Cy} + 2\text{UH}{=}\text{S} \qquad (2)$$

where the second reaction is presumably very rapid, so that the first one would be the rate-determining step, in accordance with the finding that the rate of oxidation of cysteine was independent of its concentration, being a concentration function only of the catalyst and the oxidizing agent. In addition, however, Pirie found that when cysteine reacted with an equimolar or larger amount of dithioformamidine not cystine but another compound resulted. The product of this reaction was not isolated, but on the basis of polarimetric observations and certain properties of the solution the following interpretation was given.

$$\text{Cy}{-}\text{SH} + \text{U}{-}\text{S}{-}\text{S}{-}\text{U} + \text{H}_2\text{O} \rightarrow \text{Cy}{-}\text{SOH} + 2\text{UH}{=}\text{S} \qquad (3)$$

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[1] In the present paper the following symbols are used: Cy— for $\text{HOOC}{-}\text{CH(NH}_2){-}\text{CH}_2{-}$, U— for $\text{NH}_2(\text{NH}{=})\text{C}{-}$, and UH= for $(\text{NH}_2)_2\text{C}{=}$, so that UH=S stands for the common formula of thiourea.

An experiment was also cited purporting to show that the same product will result from cystine according to

$$\text{Cy—S—S—Cy} + \text{U—S—S—U} + 2H_2O \rightarrow 2\text{Cy—SOH} + 2\text{UH=S} \quad (4)$$

although the paper contains contradictory statements on this point.[2]

The observation that the reaction product gave no color with Folin's phosphotungstic acid reagent when alkaline sulfite was added before the reagent, while a blue color resulted when the solution was made alkaline previously to the addition of sulfite, was taken as indicating that the Cy—SOH is stable in acid solution, that under alkaline conditions it reacts with sulfite according to

$$\text{Cy—SOH} + SO_3H^- \rightarrow \text{Cy—S—}SO_3^- + H_2O \quad (5)$$

while in the absence of sulfite it rapidly decomposes with formation of cystine. Also the observed formation of cystine from 1 molecule of the compound and 1 molecule of cysteine could be explained in terms of the same interpretation.

$$\text{Cy—SOH} + \text{Cy—SH} \rightarrow \text{Cy—S—S—Cy} + H_2O \quad (6)$$

This theory as a whole appears satisfactory when no preconceived ideas are held about the properties of the sulfenic acid, but it is difficult to reconcile with findings on the decomposition of cystine disulfoxide (Lavine, 1936) and especially with differently approached studies on the possible isolation of the sulfenic acid (Toennies, 1935, 1936). Consideration of the question whether the theory of Pirie is the only one consistent with his data showed that another interpretation would fit the available evidence equally well.

$$2\text{Cy—SH} + \text{U—S—S—U} \rightarrow \text{Cy—S—S—Cy} + 2\text{UH=S} \quad (7)$$

However, when dithioformamidine is present in equimolar, or larger, amount

$$\text{Cy—SH} + \text{U—S—S—U} \rightarrow \text{Cy—S—S—U} + \text{UH=S} \quad (8)$$

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[2] Pirie (1933) states on p. 1182, "[Cystine] cannot be . . . oxidised at any appreciable rate;" p. 1183, when cystine is formed first, upon addition of another molecule of dithioformamidine, "After several hours the rotation falls to the normal value for reaction B" (formation of Cy—SOH); p. 1187, "Dithioformamidine has no effect on cystine."

The product of this reaction, an asymetric disulfide of cysteine and thiourea, could react with cysteine according to

$$\text{Cy—S—S—U} + \text{Cy—SH} \rightarrow \text{Cy—S—S—Cy} + \text{UH=S} \qquad (9)$$

and the behavior with sulfite and Folin's reagent could be interpreted thus:

$$\text{Cy—S—S—U} + \text{SO}_3\text{H}^- \rightarrow \text{Cy—S—SO}_3^- + \text{UH=S} \qquad (10)$$

especially since thiourea has been reported not to reduce the reagent (Lewis and Nicolet, 1913–14; Shinohara, 1935, *b*), while in alkaline solution and in the absence of sulfite a decomposition with cystine formation would be readily conceivable. In addition, the apparently conflicting statements on the interaction between dithioformamidine and cystine could be explained by assuming a catalysis of the reaction between the two disulfides by thiourea (which in the experiment cited before is present in addition to the reactants proper).

$$\text{Cy—S—S—Cy} + \text{UH=S} \rightarrow \text{Cy—S—S—U} + \text{Cy—SH} \qquad (11)$$

$$\text{Cy—SH} + \text{U—S—S—U} \rightarrow \text{Cy—S—S—U} + \text{UH=S} \qquad (12)$$

On this basis no reaction between the two disulfides might be apparent in the absence of thiourea.

This alternate theory was fully borne out by experimental work. The postulated compound, S-(guanylthio-)*l*-cysteine,[3] $\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—S—C(=NH)NH}_2$, has been isolated as the dihydrochloride, and its formation and reactions were found to be in accordance with the mechanisms outlined.

Some related observations on the behavior of thiourea and those of its derivatives with which this paper is concerned are included in the experiment part.

EXPERIMENTAL

Reagents. Preparation of Dithioformamidine—*l*-Cysteine hydrochloride (Merck), standardized colorimetrically (Shinohara, 1935, *a*) against free cysteine (Toennies and Bennett, 1935–36), was used. Dithioformamidine dihydrochloride was prepared (*cf.* Böeseken (1936)) as follows: To a solution of 500 mM of thiourea (thiocarbamide, Merck, m.p. 177.5° corrected) in 60 cc. of 10 M HCl, 30 cc. of H_2O and 90 cc. of alcohol, cooled to less than 5°, and 25 cc. of 10 M H_2O_2 are added dropwise, the temperature being kept below 25°. After standing in the refrigerator overnight, the precipitate is filtered, washed with ether, and allowed to dry at room temperature *in vacuo*. The yield is 75 per cent of the theoretical. Titration of the hydrochloride is rendered somewhat difficult by the instantaneous precipitation of sulfur upon neutralization (*cf.* McGowan (1886), Werner (1912)).

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[3] This designation was kindly suggested by Dr. Leonard T. Capell, Associate Editor of Chemical Abstracts.

$$NH_2(NH{=})C{-}S{-}S{-}C({=}NH)NH_2 \rightarrow S + (NH_2)_2C{=}S + NH_2CN \qquad (13)$$

However, in the presence of a large amount (in order to overcome the adsorption of the indicator on the precipitated sulfur) of methyl red the theoretical equivalent weight (111.5) was obtained.

Reversible Reaction between Thiourea and Iodine—It has been shown by Werner (1912) that the reaction

$$2UH{=}S + I_2 \rightleftharpoons U{-}S{-}S{-}U + 2HI \qquad (14)$$

represents a reversible equilibrium. At high dilution the reaction is reported to go completely to the right (Reynolds and Werner, 1903). The accuracy of iodometric titration under these conditions (0.005 M thiourea) is obviously low. We have attempted to utilize instead the small solubility of dithioformamidine dinitrate (Werner, 1912). Aliquots of a 0.4 M thiourea solution (2, 4, 5, 10, 10 cc.) were combined with definite amounts, corresponding to an estimated excess of 0.5 to 0.6 milli-equivalent of iodine over the theoretical, of a 0.1 N iodine solution in 0.1 M HI and 0.8 M HNO_3 (the titer of this solution increased by 3 per cent in 1 month) and enough concentrated nitric acid to make the total concentration 0.8 M. After the solution was left at +2° overnight (in order to complete the crystallization of the nitrate), the excess iodine was *rapidly* titrated with 0.024 N thiosulfate. The point of the first disappearance of the iodine color (no starch was used) corresponded, after addition of an empirical correction of 0.073 milli-equivalent to the iodine consumed, to 102.1, 98.9, 99.3, 99.6, and 100.8 per cent (average 100 ± 1) of the theoretical amount for oxidation of thiourea to the disulfide.

The following experiments show that the reverse reaction may be used for the determination of the disulfide. The iodine liberated by 2 cc. portions of 0.04 M dithioformamidine dihydrochloride in 3 M HCl, in 12 to 13 cc. of solution containing (*a*) 2, (*b*) 3, or (*c*) 4 M KI, each in the presence of (*A*) 0.5 M HCl or (*B*) 1 M HCl, was titrated after ½ hour with 0.024 N thiosulfate (for the method of titration *cf.* Lavine (1936)), and corresponded, after subtraction of the blank value of the reagents, to 86.9 (*aA*), 93.3 (*bA*), 94.8 (*cA*) and 89.2 (*aB*), 95.3 (*bB*), 98.4 (*cB*) per cent of the theoretical amount for reduction of dithioformamidine to thiourea.

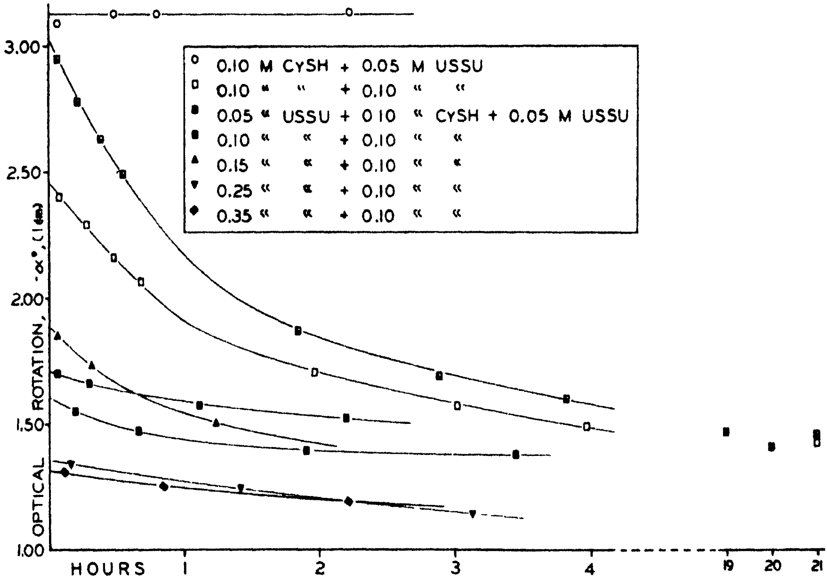

FIG. 1. The reaction between the hydrochlorides of *l*-cysteine and dithioformamidine. The reagents were added in the order given in the legend for each curve. Separation of colloidal sulfur, especially in the cases of the larger amounts of dithioformamidine, made continued accurate readings impossible.

Reactions between Cysteine and Dithioformamidine—The observations of Pirie (1933) as to the difference in the reaction end-product, depending on the ratio of dithioformamidine and cysteine, were confirmed. However, the final rotation,[4] corresponding to the reaction between equimolar amounts, was never obtained at once, even though the precaution of adding the cysteine, with vigorous shaking, to the dithioformamidine (Pirie, 1933), and not *vice versa*, was observed; but it was approached, the more rapidly the larger the excess of dithioformamidine used. This is shown in Fig. 1.

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[4] All optical rotations refer to the mercury line (5461 Å.) and a temperature of approximately 26°, unless stated otherwise.

Reaction between Cystine and Dithioformamidine—Fig. 2 shows that the rate of the reaction between the two disulfides is proportional to the thiourea concentration, in agreement with the catalytic mechanism expressed by Equations 11 and 12. However, while the reaction between the two disulfides alone was very slow at low concentrations (0.05 M), it was found that when considerably higher concentrations are used the reaction soon attains a fairly rapid rate, as is shown by the experiments illustrated in Fig. 3. The supposition that the same mechanism—with thiourea as the mediator—is responsible for this phenomenon is based on the following considerations: (*a*) the known instability of

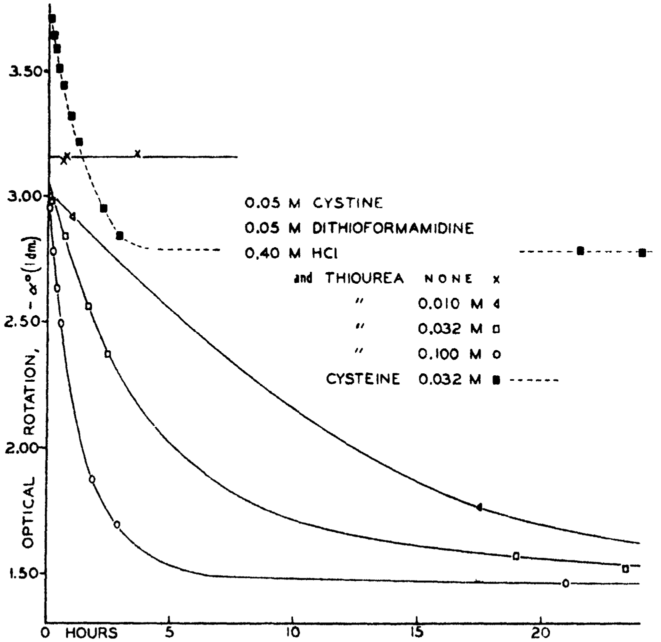

Fig. 2. The catalysis by thiourea of the reaction between cystine and dithioformamidine in acid solution.

dithioformamidine (McGowan, 1886; Werner, 1912) which even in acid solution slowly breaks down, presumably with formation of thiourea according to Equation 13 as is suggested by the appearance of free sulfur; (*b*) the fact that in the experiments of Fig. 3 the solution becomes opaque from finely dispersed sulfur as soon as the reactants have been combined (before the solutions were placed in polarimeter tubes they were clarified by shaking with filter paper pulp; the free sulfur was completely removed, but variable amounts of reactants themselves were also lost by adsorption in this procedure; sometimes filtration from newly formed sulfur was again necessary later on in order to continue the polarimetric readings); (*c*) the autocatalytic nature of the

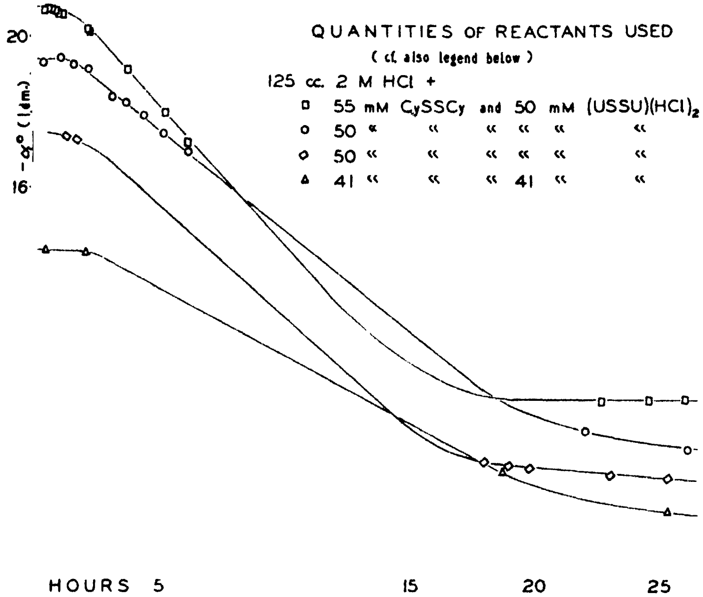

Fig. 3. The autocatalytic character of the "spontaneous" reaction between cystine and dithioformamidine in acid solution. The polarimetric values are corrected to constant temperature (28°, 25°, 27°, and 26° respectively) by use of (as an approximation) the temperature coefficient $-0.001°\alpha/+0.1°C$. (Toennies and Lavine, 1930). Their absolute magnitudes are not perfectly comparable with the quantities of reactants stated in the chart, on account of losses involved in the clarification by means of paper pulp.

early part of the curves in Fig. 3 which is as would be expected if the main reaction depends on the primary liberation of thiourea; (*d*) if the rate of the reaction is proportional to the absolute amount of thiourea present (Fig. 2 and Equations 11 and 12), and if the amount of thiourea formed from dithioformamidine is proportional to the concentration of the latter (Equation 13), it is obvious that the rate of the "spontaneous" reaction should increase with increasing initial concentration of dithioformamidine.

If Equations 11 and 12 correctly describe the reaction between the two disulfides, it is further necessary, first that the reaction is released not only by thiourea but by cysteine as well, and secondly that the reaction expressed in Equation 11 is subject to a reversible equilibrium, since the reaction postulated in Equation 9 represents the opposite process. An experiment regarding the first point has been incorporated in Fig. 2 (dotted line). The initial high optical value indicates that in the first rapid stage about 40 and 60 per cent of the cysteine have reacted according to Equations 7 and 8 respectively. The thiourea liberated hereby catalyzes, in the second slow stage (represented by the curve), the reaction between the two disulfides until a final condition is reached where only Cy—S—S—U, UH═S, and Cy—S—S—Cy, but no U—S—S—U or Cy—SH, are left. The calculated value for this condition is −2.72°, compared with the experimental final level of −2.78°.

Evidence for the second point, existence of a state of equilibrium between cystine and thiourea, was obtained in the following experiments. When a mixture of 0.25 mM of cystine and 0.25 mM of thiourea was examined for —SH by Shinohara's (1935, *a*) modification of the Folin phosphotungstic acid method, a distinct blue color resulted, while the same amounts of cystine and thiourea tested separately gave no appreciable color. It was here discovered that thiourea, while it gives no color under Shinohara's conditions, has a strong inhibiting effect on the color formation by —SH (*cf.* below). An attempt to estimate the amount of —SH formed by the interaction of cystine and thiourea was made as follows: The amount of color formed by 0.01 mM of cysteine in the presence of 2 mM of thiourea, in 25 cc., after 20 hours in the presence of the amounts of buffer and reagent specified by Shinohara, corresponded to 0.0089 mM of —SH when read against the ordinary cysteine standard. When a solution (in 1 cc. of 1.65 M HCl) of 0.4 mM of cystine (which alone gives no color) and 2 mM of thiourea was similarly tested, the color (after precipitated cystine is filtered off) corresponded to 0.0028 mM of —SH, *i.e.* to 31 per cent of the color produced by 0.01 mM of cysteine under comparable conditions. This result would indicate conversion of about 0.8 per cent of the cystine into cysteine according to Equation 11.

S-(Guanylthio-)l-Cysteine, Isolation and Analysis—20 mM of *l*-cystine and 20 mM of dithioformamidine hydrochloride are dissolved in 50 cc. of 2 M HCl. After being shaken with shredded filter paper the solution is clarified (*cf.* preceding section). When the optical rotation, which begins to decrease within 1 or 2 hours, has become constant (*i.e.* after 1 or 2 days), 2 to 3 volumes of ether are added with cooling, and the solution is saturated with hydrogen chloride at about 0°. The reaction product tends to separate as a clear oily deposit, in which crystallization is readily induced by vigorous shaking. After the material is left at low temperature overnight, the white crystalline deposit is separated by centrifuging and washed by trituration and centrifuging as follows: first with 15 cc. of isopropyl alcohol, then with 10 cc. of isopropyl alcohol plus 30 cc. of ether, and finally four times with 50 cc. of ether. The dried product (about 8 gm.), which may contain about 10 per cent of cystine, is purified by digestion with (about 3.1 cc. per gm. of substance) 14 M hydrochloric acid (saturated at 0°). An insoluble residue (chiefly cystine hydrochloride) is discarded and the reaction product is reprecipitated from the solution by addition of 3 volumes of cold ether and vigorous shaking. After the crystallization is completed (at low temperature), the product is separated and washed as outlined above, except that the final digestion with ether is repeated about twelve times in order to free the substance of hydrogen chloride. The yield is about 50 per cent of the theoretical. In order to increase the purity of the product the reprecipitation procedure may be repeated.

*Analysis**—$C_4H_{11}N_3O_2S_2Cl_2$ (268.1)

Calculated.	S 23.9,	N 15.7,	Cl 26.5
Found.	" 23.6, 23.5,	" 15.9, 15.2,	" 26.3, 25.6

* S was determined by oxidation by the alkaline permanganate method; N, by the micro-Kjeldahl method of Folin and Denis (1916); Cl, by the Volhard titration, *cf.* below on reactions with $AgNO_3$.

Equivalent weight, first end-point on rapid titration (methyl red), 268, 265; calculated 268; *cf.* properties discussed below.

Reactions of Thiourea, Dithioformamidine, and S-(Guanylthio-)l-Cysteine with Silver Nitrate—Complications in the determination of ionized chlorine in the new compound were traced to the formation of AgSCN, and as a consequence the behavior with $AgNO_3$ of all three compounds was studied. 150 mg. samples of the mixed disulfide were precipitated in 230 cc. of 1.4 M HNO_3 (in order to eliminate the possibility of precipitating silver compounds of cysteine) with 3 mM $AgNO_3$; after the mixture was heated to the boiling point and allowed to settle in the dark, the precipitate was filtered, washed, dissolved in ammonia, and, after filtration, reprecipitated under the usual slightly acid conditions. Six such determinations gave 118 to 126 mg. of silver precipitate per 100 mg. of substance which, calculated as AgCl, corresponds to 30.4 ± 0.7 per cent of Cl. However, when wet ashing (in 14 M HNO_3) and volumetric halide determination by the open Carius-Volhard procedure of Van Slyke and Sendroy (1923-24) were used, the values obtained were near the theoretical, even when heating on the steam bath ("ashing") was omitted. On the other hand, when the Volhard volumetric procedure was applied in 1 to 1.5 M HNO_3 (as in the gravimetric method), correct values resulted if the solution was not heated at all, while the results were too high, if after precipitation the solution was briefly heated to boiling as in the gravimetric method. Calculation on the basis of the gravimetric and volumetric values, and qualitative tests, showed that the high "halogen" values are due to formation of AgSCN from the disulfide. This is formed only when the (moderately acid) solution is heated briefly, while on longer heating in the presence of concentrated HNO_3 (wet ashing), it is destroyed by oxidation. The maximum amounts of AgSCN obtained (0.1 mM of (Cy—S—S—U) $(HCl)_2$, 0.5 mM of $AgNO_3$, and 20 mM of HNO_3 in 22 cc., boiled for 1 to 5 minutes) were 0.33 to 0.42 mole of AgSCN per mole of Cy—S—S—U.

When (U—S—S—U)$(HCl)_2$ was similarly treated, 0.38 and 0.42 mole of AgSCN were formed. Thiourea under the same conditions, with and without heating, formed a white precipitate which corresponded to 0.68 and 0.87 equivalent of Ag^+ respectively per mole of thiourea. This precipitate, however, contains no

AgSCN, as it completely dissolves on warming, and crystallizes on cooling in a felt of long crystalline needles. On continued heating it soon decomposes with formation of black Ag_2S. According to these properties and the conditions of its formation, it undoubtedly belongs to the series of thiourea-silver nitrate addition complexes extensively studied by Kurnakow (1891) and Reynolds (1892).

Effect of Thiourea on the —SH and —S—S— Determination by Phospho-18-Tungstic Acid—Before any reliable conclusions could be drawn from colorimetric phosphotungstate determinations in the presence of thiourea or its derivatives, it was necessary to make a quantitative study of the inhibiting effect of thiourea on the color formation. The tests were carried out in 25 cc. volumetric flasks with the concentrations of buffer, reagent, and bisulfite specified by Shinohara (1935-36). The standard contained 0.01 mм of cysteine. In the presence of equal or larger amounts of thiourea the time during which the color formed by cysteine, or cystine in the presence of bisulfite, continues to increase toward its maximum and becomes increasingly longer as the amount of thiourea present is increased. Rather than to attempt to await the maximum color formation, a definite uniform time was chosen for the determinations; namely, for the —SH determinations 25 minutes and for the —S—S— determinations 35 minutes after the addition of the reagents. The corresponding color values were obtained by plotting a series of readings covering the time of 5 to 10 minutes before and after the stated reference times of 25 or 35 minutes. At 25 minutes the rate of increase of color in the —SH determinations was still 0.8 and 4 per cent per minute in the presence of 0.05 and 0.40 mм of thiourea respectively, and similar in the —S—S— determinations. The results are summarized in Tables I and II. The cystine data in Table I are obtained after subtraction of the blank corrections, according to Table II. The bisulfite blank values which increase with increasing thiourea concentration indicate that here the inhibition by thiourea, if any, is counterbalanced by the small amount of color formed by thiourea itself, and thus gives an indication of the magnitude of this effect. The cysteine values of Table I are uncorrected and thus represent the combined effect of the inhibition and color formation by thiourea. From the data of Tables

I and II a system of smooth curves can readily be constructed which allows the approximate determination of cysteine or cystine when the amount of thiourea present is known.

TABLE I

Inhibition by Thiourea of Color Formation of Cysteine, or Cystine and Bisulfite, with Phospho-18-Tungstic Acid at pH 5.2

The figures represent percentages of the theoretical color intensity attained 25 minutes (in the case of cysteine) or 35 minutes (in the case of cystine) after addition of the reagents. Addition and amounts of reagents are in accordance with the specifications of Shinohara (1935, *a*, 1935–36). The cystine values have been corrected on the basis of the blank determinations listed in Table II.

Cysteine or cystine used per 25 cc.	Thiourea present per 25 cc.				
	0.025 mM	0.050 mM	0.100 mM	0.200 mM	0.400 mM
mM	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.01(—SH)	99	91	62	32	16
0 02(—SH)	99	79	48	26	11
0 03(—SH)		68			9
0.005(—S—S—)	90	62	35	26	14
0 010(—S—S—)	81	55	30	22	12

TABLE II

Color Formation by Combinations of Bisulfite and Thiourea with Phospho-18-Tungstic Acid at pH 5.2

The color intensity is expressed in terms of millimoles of cystine per 25 cc. The bisulfite concentration (0.06 M) and the amounts of buffer and reagent are in accordance with the specifications of Shinohara (1935–36). The blank values for 0 thiourea concentration are taken from Shinohara (1935–36).

Time after addition of reagents	Thiourea present per 25 cc.			
	0.00 mM	0.10 mM	0.20 mM	0.40 mM
min.	$mM \times 10^4$	$mM \times 10^4$	$mM \times 10^4$	$mM \times 10^4$
20	1.55	2 10	2 45	2.90
30	1 75	2 70	3.15	3 65
60	2.20	3 40	4.40	4 75

Properties of S-(Guanylthio-)l-Cysteine—The hydrochloride is extremely soluble (about 1 gm. in 3 cc.) in 14 M HCl, also very soluble in methanol, little soluble in ethanol, and practically insoluble in isopropyl alcohol. The decomposition point of preparations containing about 2 per cent of cystine is at 150–155°.

The molar rotation (0.1 M Cy—S—S—U, 0.3 M HCl) found was $[M]^{26}_{\text{Hg}} = -110°$ or 16 per cent of the corresponding cystine value (0.05 M Cy—S—S—Cy, 0.2 M HCl), compared with $[M]_{\text{Hg}} = -121°$ (or 18.5 per cent of the corresponding cystine value) according to the data of Pirie (1933). In view of the fact that the latter value is derived from the optical rotation of the solution rather than from the isolated and purified compound, the degree of agreement may be taken as additional evidence that the isolated compound represents the product of Pirie's reaction.

When 0.1 mM of the compound was combined with 0.117 mM of cysteine in 50 cc. of solution buffered to pH 5.2, the amount of —SH that disappeared after 5 and 40 minutes and the amount of reactive —S—S— formed, both as determined by the phospho-18-tungstic acid method (Shinohara, 1935, *a*, 1935–36), were 95 and 94 per cent respectively of the amounts required for the reaction expressed by Equation 9.

Available observations relating to the stability of the compound and the problem of its mode of hydrolysis are as follows: compared with equivalent amounts of cysteine, neither S-(guanylthio-)-cysteine nor thiourea or its disulfide gives any measurable amount of color in the Folin-Shinohara method for —SH or —S—S—. When 0.2 mM per 25 cc. of the mixed disulfide were tested, the disulfide color corresponded to 0.0006 to 0.0012 mM of —S—S—, presumably due to admixture of cystine.[5] On the other hand, polarimetric observation, as well as the behavior on titration, indicates rapid hydrolytic decomposition of the compound. A solution of 0.1 M (Cy—S—S—U)$(HCl)_2$ in 1.6 M sodium acetate and 0.5 M acetic acid (pH 5 to 5.2) showed 8 minutes after dissolving $\alpha_{\text{Hg}} = -2.68°$. The polarimetric reading then increased in a straight line to −3.37° after 25 minutes, gradually reaching a steady level of −3.84° after about 1 hour, when a slight opalescence of colloidal sulfur made further readings impossible. A disulfide test on a fraction of the solution now corresponded to 0.33[6] mole of —S—S— per mole of Cy—S—S—U used. On titration (methyl red indicator) the fresh compound consumes 1 equivalent of alkali per molecule (molecular weight 268); after it had stood, and more rapidly when heated, the acidity reappears, until after several hours heating on the steam bath the total alkali consumed corresponds to 2 equivalents per molecule (equivalent weight 134). Only small amounts of colloidal sulfur appear during the decomposition. Further, the fresh compound (0.011 M $(\mathrm{Cy{-}S{-}S{-}U})(\mathrm{HCl})_2$ in 1 M HCl and 3 M KI) liberated after 2 hours 91 per cent of the amount of iodine, corresponding to the equation

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[5] If in the test the compound reacts with bisulfite according to Equation 10, the actual amount of cystine present will be about 4 times as large as indicated, owing to interference of the thiourea (*cf.* Table I) formed.

[6] Corrected for interference by thiourea (*cf.* Table I), assuming that 1 molecule of UH═S results from each molecule of Cy—S—S—U, the figure would be 0.36.

$$\begin{gathered}2\mathrm{Cy{-}S{-}S{-}U} + 2\mathrm{H_2O} + 2\mathrm{I}^- \rightarrow \\ 2\mathrm{UH{=}S} + \mathrm{Cy{-}S{-}S{-}Cy} + \mathrm{I_2} + 2\mathrm{OH}^- \end{gathered} \qquad (15)$$

which shows a reduction comparable with that of dithioformamidine under similar conditions (*cf.* above; conditions under which cystine is not reduced), while after the polarimetrically observed decomposition had come to equilibrium with the formation of about 33 moles per cent of colorimetrically reactive disulfide, no iodine was liberated under the same conditions.

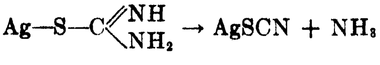
While complete description of the spontaneous decomposition of the compound must be deferred until the reaction products have been completely identified, the available evidence indicates that the first step consists in a hydrolysis of the disulfide linkage according to

$$\mathrm{Cy{-}S{-}S{-}U} + \mathrm{H_2O} \rightarrow \mathrm{Cy{-}SOH} + \mathrm{U{-}SH} \qquad (16)$$

rather than according to the alternative possibility

$$\mathrm{Cy{-}S{-}S{-}U} + \mathrm{H_2O} \rightarrow \mathrm{Cy{-}SH} + \mathrm{U{-}SOH} \qquad (17)$$

for the following reasons. In the reaction with $\mathrm{AgNO_3}$ no Cy—SAg (to be expected according to Equation 17) is formed, but AgSCN in an amount similar to that formed from U—S—S—U under the same conditions. If AgSCN be assumed to result from a breakdown of the primarily formed mercaptide of pseudothiourea,

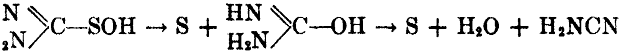


$$\mathrm{Ag{-}S{-}C}\begin{matrix}\nearrow \mathrm{NH} \\ \searrow \mathrm{NH_2}\end{matrix} \rightarrow \mathrm{AgSCN} + \mathrm{NH_3} \qquad (18)$$

similar amounts of the latter are likely to be formed in both cases: from Cy—S—S—U according to Equation 16 and from U—S—S—U according to

$$U—S—S—U + H_2O \rightarrow U—SOH + U—SH \qquad (19)$$

Further, the results of McGowan (1886) and Werner (1912) on the neutral decomposition of U—S—S—U, which are in agreement with the principle of Fromm (1906) on the hydrolysis of disulfides with adjoining double bonds, show that U—SOH spontaneously decomposes as follows:



$$\begin{matrix} N \\ {}_2N \end{matrix}\!\!>\!C—SOH \rightarrow S + \begin{matrix} HN \\ H_2N \end{matrix}\!\!>\!C—OH \rightarrow S + H_2O + H_2NCN \qquad (20)$$

so that the absence of significant amounts of sulfur in the decomposition of Cy—S—S—U may again be taken as evidence against hydrolysis according to Equation 17. Finally, in the phospho-18-tungstic acid method for —S—S— not only does Cy—S—S—U give no color, but in addition its spontaneous decomposition in which about 33 moles per cent of colorimetrically reactive —S—S— are otherwise formed is prevented. This is in entire agreement with a reaction with bisulfite according to Equation 10 which, of course, is analogous to the reaction of Equation 16 as far as the polarity of the two moieties is concerned.

$$Cy—S—S—U \rightarrow Cy—S^+ + {}^-S—U \qquad (21)$$

DISCUSSION

As was pointed out by Pirie (1933) the physiological importance of the relations between the cysteine-cystine system and thiourea may not rest so much with thiourea itself as possibly with the thiourea derivative ergothioneine.

Recent findings of Brand and coworkers (1935), on the cystinuric and of Medes (1937) on the normal organism suggest that the oxidative metabolism of cysteine may not proceed by way of cystine. These results find their counterpart in experiments on tissue slices by Pirie (1934) which indicate that formation of sulfate from cystine may be preceded by conversion of the latter into cysteine. This conversion may be an (enzymatic) hydrogenation (reduction) in the ordinary sense or it may be a hydrolysis according to the scheme —S—S— + H_2O → —SH + —SOH, where the lability of the —C—S— linkage in the —SOH derivative, suggested by the work of Schöberl (1933) and Schöberl and Eck (1936) may be an important factor in the oxidation to sulfate. Another possibility would consist in a metathesis between cystine and ergothioneine (or a similar compound) in a manner analogous to Equation 11 in which the Cy—S—S—U analogue would hydrolyze, according to Equation 16, into Cy—SOH and the parent substance (ergothioneine).

The mixed disulfide described in the present paper represents a combination of two —SH compounds of evidently quite different reducing potentials, cysteine and (pseudo-)thiourea, in which cysteine appears to be the more strongly reducing component as indicated by the relative behavior of the two compounds and the respective disulfides with phospho-18-tungstic acid and with iodine and iodide, and in which, on hydrolysis, the cysteine moiety tends toward the positive charge (Cy—S^+ + $^-$S—U). Compounds of this general type might conceivably play a rôle, by way of the reaction suggested in Equation 11, in the interaction of different —SH—S—S— systems (*cf.* Hopkins (1925), du Vigneaud *et al.* (1931-32), Goddard and Michaelis (1934), Mirsky and Anson (1935)). Brand and his associates (1936) have recently presented evidence for the formation of a mixed disulfide under physiological conditions, and Lavine (1936) has shown the possibility of their formation from cystine disulfoxide and thiol compounds.

The skilful analytical help of Mr. Thomas P. Callan is acknowledged with appreciation.

SUMMARY

The metathesis of dithioformamidine and cysteine to thiourea and S-(guanylthio-)cysteine has been demonstrated. The latter compound has been isolated as the dihydrochloride and evidence has been adduced which indicates its tendency to hydrolyze into thiourea and the sulfenic acid corresponding to cysteine. The conclusion, supported by direct evidence, has been reached that a reversible equilibrium may exist between cystine and thiourea on the one side and cysteine and S-(guanylthio-)cysteine on the other.

BIBLIOGRAPHY

Böeseken, J., *Proc. Acad. Sc. Amsterdam*, **39**, 717 (1936).

Brand, E., Block, R. J., Kassel, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).

Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).

Folin, O., and Denis, W., *J. Biol. Chem.*, **26**, 473 (1916). *Cf.* Folin, O., Laboratory manual of biological chemistry, New York and London, 4th edition, 131 (1925).

Fromm, E., *Ann. Chem.*, **348**, 144 (1906).

Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, **106**, 605 (1934).

Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).

Kurnakow, N., *Ber. chem. Ges.*, **24**, 3956 (1891).

Lavine, T. F., *J. Biol. Chem.*, **113**, 583 (1936).

Lewis, H. B., and Nicolet, B. H., *J. Biol. Chem.*, **16**, 369 (1913–14).

McGowan, G., *J. Chem. Soc.*, **49**, 190 (1886).

Medes, G., *Biochem. J.*, in press (1937).

Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **18**, 307 (1935).

Pirie, N. W., *Biochem. J.*, **27**, 1181 (1933); **28**, 305 (1934).

Reynolds, J. E., *J. Chem. Soc.*, **61**, 249 (1892).

Reynolds, J. E., and Werner, E. A., *J. Chem. Soc.*, **83**, 1 (1903).

Schöberl, A., *Ann. Chem.*, **507**, 111 (1933).

Schöberl, A., and Eck, H., *Ann. Chem.*, **522**, 97 (1936).

Shinohara, K., *J. Biol. Chem.*, **109**, 665 (1935, *a*); **110**, 263 (1935, *b*); **112**, 683 (1935–36).

Toennies, G., read before the Division of Biological Chemistry at the Eighty-ninth meeting of the American Chemical Society at New York (1935); and the Ninety-second meeting at Pittsburgh (1936).

Toennies, G., and Bennett, M. A., *J. Biol. Chem.*, **112**, 497 (1935–36).

Toennies, G., and Lavine, T. F., *J. Biol. Chem.*, **89**, 153 (1930).

Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **58**, 523 (1923–24). *Cf.* Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 829 (1932) and Kolthoff, I. M., and Menzel, I. H., Volumetric analysis, Practical volumetric analysis, New York and London, 227 (1929).

du Vigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, **94**, 233 (1931–32).

Werner, E. A., *J. Chem. Soc.*, **101**, 2166 (1912).

A CHROMOGENIC TUNGSTATE AND ITS USE IN THE DETERMINATION OF THE URIC ACID OF BLOOD

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While studying the complex tungstic acids that are the active constituents of the uric acid reagents (20–22, 3, 31), we found that it was a simple matter to make a lithium salt of a highly chromogenic arsenotungstic acid. We condensed sodium tungstate and arsenic pentoxide, following the system Wu (35) used to form 1:18 phosphotungstic acid, and found that the exceedingly active complex which formed was precipitated from solution when it was added to lithium chloride. The aqueous solution of this active salt is a uric acid reagent, uncontaminated by the mass of chromogenically inert acids and salts which are present in the uric acid reagents as ordinarily prepared.[1] This reagent is far less acidic than the other reagents, and its reduction can be observed over a wide range of pH. We undertook a study of its reaction with uric acid hoping that in properly buffered alkaline solutions reduction of the reagent by compounds other than uric acid could be excluded. Thus a direct method for uric acid might have been developed. However, extensive research with this object in mind has led us to the conclusion that all direct methods for uric acid in which these complex tungstic acids are used as reagents are unreliable when used on blood filtrates containing the total non-protein nitrogen. We put forward our reagent with the stipulation that it is to be used only on suitably fractionated biological liquids.

The lithium arsenotungstate reagent we consider preferable to other uric acid reagents for the following reasons. It gives no color whatsoever in the blank. The color of the reduced reagent is a "pure" blue.[2] The reaction is carried on at room temperature and develops promptly. The color developed by pure solutions of uric acid, by the method described below, is proportional to the amount of uric acid present. We have checked this by measurement of light transmission with a photoelectric colorimeter of the conventional fixed layer type and the photoelectric colorimeter designed in this laboratory by Goudsmit and Summerson (27). Since the sensitivity of our reagent used with cyanide holds for 0.001 to 0.007 mg. of uric acid, it is possible to estimate amounts found in 0.1 cc. of blood. The reagent appears to keep indefinitely. Samples prepared and diluted for use in blood work over 2 years ago are still as good as freshly prepared lots. Quantities of the salt may be prepared, preserved in a dry state, and dissolved in water whenever the reagent is needed. As would be expected, the reagent has much less tendency to cause turbidity than the usual reagents. However, the active constituent of our reagent is a turbidity-producing substance, so that recrystallization of the salt with its resultant purification does not by any means eliminate this troublesome factor.

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[1] The reagent of Jackson and Palmer (28) is an exception. It is composed of two active fractions separated from the Folin reagent.

To overcome this tendency toward turbidity we have found it necessary to include urea[3] in the cyanide used in blood determinations where total volume of solution is small and where careless overoxalation is frequently encountered. With the use of the cyanide-urea solution described below, we have never had a blood filtrate that showed the slightest opalescence or developed turbidity. As a result, unhurried, duplicate readings are possible. The proportionality holds over many hours. We have actually observed that filtrates determined one afternoon were perfectly clear the next morning and when reread were found to have changed less than 8 per cent. Like all cyanide solutions, this cyanide-urea solution deteriorates. By the time these solutions are 2 months old they usually contain traces of reducible material which will affect the reagent. If deterioration has reached a stage where color develops in a blank, fresh cyanide should be prepared. Owing to impurities in cyanides, there is some variation in the amount of color different lots of cyanide develop with the same amount of reduced reagent. Therefore color in standard and unknown should be developed with the same lot of cyanide.

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[2] Blanks such as are familiar to workers who have used the older reagents can be obtained from the filtrate which we discard in preparing our reagent. With phenols, colors ranging from yellow through green to purple are given by the traces of chromogenic material in these filtrates. Similar traces of chromogenic by-products probably cause the irregularities in shades of blue obtained with the ordinary reagents.

[3] For the same reason Folin introduced the use of urea in 1930 (18).

Method for Determination of Uric Acid in Blood

Solutions Required—

(*a*) Solutions for precipitation of blood proteins (10, 5).

1. Molybdotungstate solution. Boil 10 gm. of pure ammonia-free molybdic acid with 50 cc. of N sodium hydroxide for 4 to 5 minutes. Filter, and wash the filter with 150 cc. of hot water. Cool the total filtrate and mix with a solution of 80 gm. of sodium tungstate dissolved in 600 cc. of water. Dilute to 1 liter.

2. 0.62 N sulfuric acid.

To precipitate the proteins of the blood 1 volume of blood laked with 7 volumes of water is treated with 1 volume of molybdotungstate solution, followed by 1 volume of sulfuric acid.

(*b*) Solutions for separating uric acid from glutathione and thioneine (6).[4]

1. A solution containing 7.5 gm. of lithium chloride and 35 cc. of concentrated hydrochloric acid per liter.

2. 2.9 per cent silver nitrate solution.

(*c*) Standard uric acid solution.

Stock phosphate standard (9) diluted so that 5 cc. contain 0.02 mg. of uric acid; prepared by diluting 10 cc. of stock solution with water, adding 3.5 cc. of concentrated hydrochloric acid, and diluting to 500 cc.

(*d*) Solutions for developing color.

1. Alkaline solution. 5 per cent sodium cyanide solution containing 20 per cent urea.

2. Uric acid reagent prepared as follows: 100 gm. of sodium tungstate are dissolved in 500 cc. of water and 140 gm. of arsenic pentoxide are added to the solution. This mixture is boiled under a reflux condenser for 1 hour. The condenser is

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[4] This fractionation procedure (adapted for our requirements) is identical in theory with that worked out by Benedict and Behre (6) and depends on the precipitation of thioneine and glutathione by silver in acid solution.

then removed and the boiling continued until the volume of the solution is about 200 cc. This solution is slowly poured with stirring onto 100 gm. of lithium chloride. The stirring is continued until all of the white lithium chloride has gone into solution and then the mixture is chilled thoroughly (at least to 10°) for about 2 hours.[5] The lithium compound of arsenotungstic acid, by this time well settled, is filtered off on a Buchner funnel and dried as completely as possible by suction.[6] The precipitated salts should weigh about 130 gm. They are dissolved in water and made up to 500 cc. This stock solution of reagent is diluted 1:5 for blood determination.

Along with the slightly greenish chromogenic salt, there is precipitated a white non-chromogenic compound which accounts for about one-third of the total weight. It can be separated from the active salt by dissolving the active salt in alcohol. The active salt can be recovered by careful evaporation of the alcohol at low temperature and its aqueous solution can be used as the uric acid reagent. However, we consider this utterly unnecessary and, in fact, undesirable, since loss of activity is apt to occur when these sensitive salts are subjected to too much treatment. The white non-chromogenic compound we have not investigated thoroughly, but it seems to in no way adversely influence the reagent as used. It gives no color in a blank, nor does it give color with uric acid, nor is it a factor which causes turbidity.

Procedure

Mix 5 cc. of 1:10 molybdotungstic acid blood filtrate in a 15 cc. centrifuge tube with 1 cc. of acid lithium chloride. Add 1 cc. of silver nitrate and shake well. Centrifuge at once and pour the supernatant liquid into a test-tube, allowing time for complete drainage.[7] Transfer 5 cc. of the standard uric acid containing

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[5] Longer chilling is inadvisable as molybdic compounds, formed from molybdenum which may have been present as impurity in the sodium tungstate used to prepare the reagent, may precipitate and contaminate the final product. If our directions are followed, the active fraction shows no trace of molybdenum when tested with potassium xanthate (Folin and Trimble (24)).

[6] If strong suction is not available, the bulk of the liquid clinging to the precipitate should be pressed out between filter papers.

[7] After the bulk of the liquid has been poured into the test-tube in which the determination is to be made, we have found it convenient to leave the centrifuge tube inverted over the test-tube while the last drops drain out. This is done by supporting the pointed end of the inverted centrifuge tube against a notched board, the open end of the centrifuge tube resting against the inside edge of the test-tube. Clamps in ring stands hold the notched

0.02 mg. of uric acid to another tube and add 2 cc. of water.[8] Add 3 cc. of cyanide (poison, *burette always*) and 1 cc. of dilute lithium arsenotungstate reagent (poison) to each tube and mix by inversion. Read after 10 minutes.

The reading of the standard divided by the reading of the unknown is multiplied by 4 to obtain the mg. of uric acid per 100 cc. of blood. Readings between 10 mm. and 30 mm. against the standard at 15 mm. are reliable.

TABLE I

Uric Acid Content of Human Blood by New Procedure and by Folin-Wu Fractionation ((25) p. 100) Followed by Benedict Method

The figures represent mg. of uric acid per 100 cc. of blood (molybdotungstate precipitation).

Sample No.	New method	Folin-Wu fractionation followed by Benedict method	Sample No.	New method	Folin-Wu fractionation followed by Benedict method
1	2.55	2.53	14	4.28	4.31
2	3.37	3.24	15	4.25	4.13
3	4.09	3.85	16	4.20	4.25
4	3.17	3.07	17	3.75	3.52
5	3.36	3.14	18	3.45	3.35
6	3.77	3.70	19	3.37	3.37
7	4.54	4.19	20	4.10	3.82
8	3.63	3.68	21	5.16	5.42
9	3.66	3.63	22	3.75	3.37
10	3.70	3.37	23	3.00	2.81
11	3.57	3.35	24	2.84	2.49
12	3.04	3.19	25	3.50	3.16
13	3.72	3.77	26	3.41	3.32

Using this procedure we have made determinations on twenty-six human blood samples and compared them with figures obtained by the Benedict (3) method on the sodium chloride-hydro-

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board in a position parallel to the test-tube rack, about 1½ inches behind and 3 inches above the tops of the test-tubes placed in the rack. Our board was formed by cutting the top board of a test-tube rack lengthwise through the perforations. After 1 minute the last drop of liquid clinging to the lip of the centrifuge tube is removed with the help of a glass rod and added to the fraction in the test-tube.

[8] Workers wanting more than 11 cc. for rechecking standards should of course double all measurements.

TABLE II

Recovery of Uric Acid Added to Blood (Molybdotungstate Precipitation)

Sample No.	Untreated blood	Blood to which 4 mg. uric acid per 100 cc. have been added (in laking water)	Recovery
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
1	3.16	6.85	92.2
	3.19	6.96	94.2
2	3.36	6.94	89.5
	3.22	6.97	93.7
3	3.70	7.19	87.2
	3.84	7.21	84.2
4	3.70	7.14	86.0
5	3.57	7.09	88.0
6	3.72	7.21	87.2
7	4.20	7.69	87.2
8	3.75	7.11	84.0
9	3.44	7.35	97.7
10	3.37	7.19	95.5
11	4.10	8.26	104.0
12	5.16	8.92	94.0
13	3.75	7.25	87.5
14	3.00	6.25	81.2
15	2.84	6.47	90.7
16	3.50	7.63	103.2
17	3.40	6.94	88.5
Average			90.8

TABLE III

Figures Showing Separation of Thioneine from Uric Acid

The values are expressed as mg. of uric acid per 100 cc.

Filtrate	Untreated blood	Blood to which 10 mg. thioneine per 100 cc. have been added (to filtrate)
Molybdotungstate	3.75	3.75
Tungstate	3.70	3.66

chloric acid-soluble fraction of the silver lactate precipitate of Folin and Wu ((25) p. 100). The figures by the two procedures recorded in Table I agree fairly closely.

Recoveries of uric acid added to the laking water used in obtaining the blood filtrates average by the new method 90.8 per cent (Table II).

TABLE IV

Recovery of Uric Acid after Addition of Thioneine As Well As Uric Acid

The values are expressed as mg. of uric acid per 100 cc. (molybdotungstate precipitation).

Sample No.	Untreated blood	Blood to which 4 mg. uric acid per 100 cc. have been added (in laking water)	Per cent recovery	Blood to which 4 mg. uric acid + 10 mg. thioneine per 100 cc. have been added (in laking water)	Per cent recovery
1	3 36	6.94	89.5	7 18	95 5
		6 97	90 2		
2	4 20	7 69	87 2	7 69	87 2
3	3 50	7 63	103 2	7.35	96 2

TABLE V

Figures for Uric Acid after Fractionation by Two Procedures after Addition of Glutathione (50 Mg. per 100 Cc. of Blood)

The figures are expressed as mg. of uric acid per 100 cc. of blood (molybdotungstate filtrate).

Fractionation	Blood filtrate	Blood filtrate + glutathione
New method	3 45	3 42
Folin-Wu followed by Benedict method	3.18	3 29

TABLE VI

Recovery of Uric Acid Added to Laking Water When Blood Proteins Are Precipitated by Either Tungstic Acid or Molybdotungstic Acid

Filtrate	Blood	Blood + 4 mg. uric acid	Recovery
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
Molybdotungstic acid	3.41	6.94	88.2
Tungstic acid	3 39	7.14	93.7

That the separation of uric acid from thioneine is satisfactory in the new procedure is shown by the figures for uric acid after thioneine has been added to the blood (Table III) and by the recoveries of uric acid when thioneine as well as uric acid has been added (Table IV).

Figures for uric acid remain practically constant after fractionation by the two procedures when glutathione has been added to the filtrates in amounts corresponding to 50 mg. per 100 cc. of blood (Table V).

The recoveries of uric acid added to water used to lake the blood are satisfactory whether the proteins are precipitated by the tungstic acid method of Folin and Wu ((25) p. 82) or by the molybdotungstic acid procedure (10, 5) (Table VI).

DISCUSSION

The lithium arsenotungstate reagent is only mildly acidic and uric acid reduces it in the presence of many weak and strong alkalies. We hoped that we could develop a method in which cyanide was unnecessary. But we found that when determinations are to be made on blood filtrates where 0.02 mg. of uric acid is the usual standard, the amount of color developed is not sufficient for good readings, nor is the proportionality good, unless cyanide with its "induced reduction" effect is used. This property was first noted by Benedict and Hitchcock in 1915 (9) when they used cyanide to dissolve the ammoniacal silver urate precipitated in their procedure for urine, and in 1922 Morris and Macleod (31), Jackson and Palmer (28), and Benedict (3) all published blood methods in which cyanide replaced other alkalies previously used. Cyanide has the effect of lessening interference from other substances in the filtrates, because of the tremendously increased color it produces with uric acid, an increase not paralleled by the other substances. This property has been the basis of many direct methods.

It is unnecessary to point out all the advantages of a direct procedure. Simplicity, speed, and elimination of losses apt to occur in fractionation come immediately to mind. However, our study with this new reagent has led us to abandon all direct methods and we feel strongly that fractionation with its possible losses and its attendant "bother" is to be preferred to any procedure in which more than traces of interfering reactive substances are present in the solution in which the color is to be developed. The blood constituents, thioneine, glutathione, and ascorbic acid, all affect these complex tungstates. Thioneine not only reduces our reagent but in the presence of glutathione interactions occur so that it can give as much as twice its real value, as expressed in uric acid color (Table VII). The amount of glutathione in 5 cc. of a 1:10 blood filtrate does not reduce our reagent, as used, but if the thioneine of the filtrate has not been separated from the glutathione of the filtrate, more reduction takes place and the resultant color is greater than the amount of thioneine present would produce if in pure solution. Thus it becomes clear, since glutathione is always present in the so called "thioneine fraction" (the silver residue left after fractionating blood for uric acid determination by either the Folin-Wu ((25) p. 100) silver lactate procedure or the Benedict-Behre (6) method), that thioneine methods (1, 12) which depend on dissolving these silver salts in cyanide, treating with uric acid reagents, and developing the color with alkali give incorrect figures for thioneine. This catalytic effect of glutathione is similar to interactions discussed by Benedict and Gottschall (8) and holds for all combinations of reagents and alkalies we have tried.

TABLE VII

Solution of Thioneine Compared with Solution of Thioneine Plus Glutathione by New Method

	Reading
	mm.
0.05 mg. thioneine	20
0.05 " " + 0.03 mg. glutathione	13
0.05 " " + 0.05 " "	10.1
0.05 " " + 0.10 " "	7.5
0.05 " " + 0.20 " "	6.8
0.05 " " + 0.30 " "	5.1
0.10 mg. thioneine	20
0.10 " " + 0.20 mg. glutathione	8
0.2 mg. glutathione	Colorless

The Folin-Wu acid silver lactate precipitate carries down most or all of the uric acid ((25) p. 100), glutathione (11), and thioneine (12) of the blood filtrates and this precipitate extracted with

NaCl-HCl solution yields a solution containing most or all of the uric acid ((25) p. 100) and minimal amounts of the other compounds (11). The precipitation of the glutathione and thioneine of these filtrates by silver nitrate in a solution made acidic with HCl and of increased chloride content, as worked out by Benedict and Behre (6) and adapted by us for use with our reagent, is less laborious and under ordinary circumstances apparently as accurate. However, it is easy to demonstrate that ascorbic acid might contribute to the color developed in the uric acid fraction of the Benedict-Behre method. We have added ascorbic acid to the water used in laking sheep blood,[9] precipitated the proteins by the Folin-Wu ((25) p. 82) tungstic acid and the Benedict-Newton (10, 5) molybdotungstic acid procedures, and, developing uric acid color by our method, compared the uric acid figures with those of the blood to which no ascorbic acid has been added. Table VIII shows that in almost every case in which ascorbic acid has been added, the uric acid fraction of the lithium chloride-silver nitrate procedure shows considerable color, which does not appear when the silver lactate-acid sodium chloride fractionation procedure is used. To cause the same reduction the Folin method (19, 20) requires about 20 times as much ascorbic acid as uric acid, the Benedict-Behre method (6) about 6 times as much, and ours about 8 times as much. With our reagents, as used, a blood filtrate containing 4 mg. of uric acid and 4 mg. of ascorbic acid per 100 cc. of blood would give a figure about 10 per cent higher than the true uric acid figure. Since such large amounts of ascorbic acid have never been reported in these filtrates and approached in tungstic acid filtrates only after massive doses have been administered and after the technique of Taylor, Chase, and Faulkner (33) has been employed, it would appear that ascorbic acid interference may be considered negligible in ordinary blood work. However, when determinations are made on bloods in which abnormalities of vitamin C content are suspected, the Benedict-Behre method of fractionation should be checked against the Folin-Wu procedure. The same check should be made when evidence of hitherto unknown constituents of blood has been brought forward.

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[9] For this determination we chose sheep blood, since it contains minimal amounts of uric acid. As a result the reduction due to the added ascorbic acid is more apparent as it is a larger fraction of the total reduction. With normal human blood ascorbic acid interference would ordinarily be almost negligible.

It has frequently been contended (14, 19, 34) that the presence of silver depresses color developed by uric acid. We would like to call attention to figures in the last column of Table VIII which show that, by our method, determination of the total color of filtrates containing *all* of the silver nitrate used for fractionation, as well as the LiCl-HCl, yields figures closely checking those developed by untreated filtrates even when there is evidence that the filtrates contain large amounts of the highly reactive substance, ascorbic acid. The minimal amounts of silver leaking into the uric acid fractions, as used, may certainly be regarded as totally without effect on the reduction caused by uric acid.

TABLE VIII

Reducing Material in Sheep Blood before and after Addition of Ascorbic Acid Determined As Uric Acid by New Method

	Filtrate No.	Ascorbic acid added to blood in laking water	Reducing material expressed as mg. uric acid per 100 cc.						
			Total color of filtrate	Total silver lactate precipitate	Folin-Wu fractionation		LiCl-HCl and $AgNO_3$ fractionation		Total color of filtrate containing LiCl-HCl and $AgNO_3$
					Uric acid fraction	Silver residue	Uric acid fraction	Silver residue	
		mg.							
Tungsto-molybdic acid	1	None	0.80	0.47	<0.1	0.51	0.21	0.46	0.79
	2	2	0.93	0.55	<0.1	0.53	0.26	0.48	0.88
	3	6	0.98	0.53	<0.1	0.57	0.38	0.45	0.96
	4	12	1.38	0.59	<0.1	0.57	0.81	0.48	1.30
Tungstic acid	6	None	0.25	<0.2	<0.1	<0.23	<0.23	<0.23	0.26
	7	2	0.30	<0.2	<0.1	<0.23	<0.23	<0.23	0.31
	8	6	0.38	0.23	<0.1	<0.23	0.26	<0.23	0.36
	9	12	0.56	0.26	<0.1	<0.23	0.38	<0.23	0.63

That procedures giving essentially correct figures for uric acid of human blood are not necessarily applicable to animal bloods has been amply discussed by Benedict (4). Benedict and Behre (6) reemphasized this when they proposed their acid lithium chloride-silver nitrate fractionation procedure. Figures on beef blood with its high content of combined uric acid (2) illustrate this point. The uric acid fraction of a 1:5 filtrate from beef blood yields figures far too high for its content of free uric acid, owing to combined uric acid not entirely precipitated by the silver. We have satisfied ourselves as to the presence of combined uric acid in both the uric acid fraction and the silver residue of the Benedict-Behre procedure by determining uric acid before and after hydrolysis (finding increased reduction after hydrolysis) (2) and by furfural tests for pentose (ribose) (16) which were so strong that they could not be due to traces of ascorbic acid (15, 32).

From 1300 cc. of pig blood precipitated 1:5 with molybdotungstic acid and fractionated with lithium chloride and silver nitrate exactly as in our procedure (followed by hydrochloric acid extraction and mercury precipitation) we have isolated crystalline thioneine hydrochloride in an amount that suggests improvement on our old procedure (12) as regards yield. We have not investigated this thoroughly, but it serves to corroborate our contention that the thioneine of the blood is contained in the silver precipitate of our fractionation procedure used on molybdotungstate filtrates.

A few words should be said about the substitution of our reagent for other so called uric acid reagents in colorimetric studies of other reducing substances. Workers in these fields will find that the flexibility of this weakly acidic solution makes it adaptable for many colorimetric determinations in which the final blue solution measured is produced by the reduction of a chromogenic tungstate. The reducing action of ascorbic acid on such complexes is well known and has been used for several quantitative methods (26, 30). The lithium arsenotungstate is reduced by ascorbic acid in acid solution. We have for years used it as a rough check on the amount of ascorbic acid in acidified urines.

In the Benedict-Hitchcock (9) determination for uric acid of urine, which we consider the most reliable method, we use 2 cc. of the lithium reagent in place of the Folin-Denis reagent (21). The presence of vitamin C and other reacting substances makes it imperative to use an indirect procedure for uric acid in these determinations (see (7, 13, 14)). In connection with the Benedict-Hitchcock silver precipitation, we would again like to bring up the subject of interference from reduced silver. We have determined uric acid in urines before and after large amounts of ascorbic acid have been added. Ammoniacal silver precipitates from these urines are dark, showing the presence of reduced silver, but this does not result in any marked depression when the reducing material of the silver precipitates is determined. Table IX shows that the figures closely check those of the untreated urines.

As the reagent gives a negligible color with tyrosine ((23) p. 109) in pure solution, it can be adapted for use in cystine determinations ((23) p. 103, (29)) on protein hydrolysates. A fine strong blue is obtained when 1 mg. of cystine (after reduction with sodium sulfite) is treated with carbonate followed by the reagent.

Glutathione in pure solution determined by the Benedict-Gottschall (8) method gives about the same amount of color with 1 cc. of our dilute reagent as with 0.5 cc. of the Benedict reagent.

TABLE IX

Mg. of Uric Acid per 100 Cc. of Urine by Benedict-Hitchcock Method

	Urine*	Urine + 0.2 mg. ascorbic acid per 1 cc. urine†
Total color	22.7	44.3
Ammoniacal silver precipitate	16.7	16.4
	16.6	16.4

* This sample showed presence of vitamin C.

† This amount of ascorbic acid is comparable to figures for vitamin C we obtained by dichlorophenol indophenol titration (17) in normal urine after ingestion of the usual "glass of orange juice for breakfast."

The same is true of thioneine in pure solution when hydroxide is used as alkali, as in the Behre-Benedict method (1).

By procedures similar to that used in preparing our reagent, we have separated active fractions from the phosphotungstic reagent of Folin (20) and from the arsenophosphotungstic reagent of Benedict (3). In each case the reagent from 100 gm. of sodium tungstate is concentrated to about 200 cc. and added to 100 gm. of lithium chloride. The precipitate that forms contains most of the desired reactive material and contaminating molybdenum compounds remain in solution. As is the case with the lithium arsenotungstic reagent, there is precipitated white non-chromogenic material which can be separated from the active material by taking advantage of its insolubility in alcohol. A comparable amount of the lithium phosphotungstate gives less than 60 per cent as much color as our reagent. The more complicated arsenophosphotungstic product gives about 15 per cent more color than our reagent. Increase in color of this order would appear to have little advantage in determinations such as we have outlined. However, we are investigating the possibilities of the arsenophosphotungstate.

SUMMARY

The preparation of a new salt of a highly chromogenic arsenotungstate is described. A method in which this compound is used as a reagent in determining the uric acid of blood is outlined and its advantages discussed. The need for separating uric acid from other reactive substances in blood filtrates is stressed.

The possibility of adopting the reagent for the reactive chromogenic agent used in determining uric acid of urine, ascorbic acid, cystine, glutathione, and thioneine is mentioned.

Similar active complexes from the Folin phosphotungstate reagent and from the Benedict arsenophosphotungstate have been prepared.

BIBLIOGRAPHY

1. Behre, J. A., and Benedict, S. R., *J. Biol. Chem.*, **82,** 11 (1929).
2. Benedict, S. R., *J. Biol. Chem.*, **20,** 633 (1915).
3. Benedict, S. R., *J. Biol. Chem.*, **51,** 187 (1922).
4. Benedict, S. R., *J. Biol. Chem.*, **64,** 215 (1925).
5. Benedict, S. R., *J. Biol. Chem.*, **92,** 135 (1931).
6. Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, **92,** 161 (1931).
7. Benedict, S. R., and Franke, E., *J. Biol. Chem.*, **52,** 387 (1922).
8. Benedict, S. R., and Gottschall, G., *J. Biol. Chem.*, **99,** 729 (1932–33).
9. Benedict, S. R., and Hitchcock, E.H., *J.Biol. Chem.*, **20,** 619 (1915).
10. Benedict, S. R., and Newton, E. B., *J. Biol. Chem.*, **83,** 357 (1929).
11. Benedict, S. R., and Newton, E. B., *J. Biol. Chem.*, **83,** 361 (1929).
12. Benedict, S. R., Newton, E. B., and Behre, J. A., *J. Biol. Chem.*, **67,** 267 (1926).
13. Christman, A. A., and Mosier, E. C., *J. Biol. Chem.*, **83,** 11 (1929).
14. Christman, A. A., and Ravwitch, S., *J. Biol. Chem.*, **95,** 115 (1932).
15. Cox, E. G., Hirst, E. L., and Reynolds, R. J. W., *Nature*, **130,** 888 (1932).
16. Davis, A. R., Newton, E. B., and Benedict, S. R., *J. Biol. Chem.*, **54,** 595 (1922).
17. Emerie, A., and van Eekelen, M., *Biochem. J.*, **28,** 1153 (1934).

18. Folin, O., *J. Biol. Chem.*, **86,** 179 (1930).
19. Folin, O., *J. Biol. Chem.*, **101,** 111 (1933).
20. Folin, O., *J. Biol. Chem.*, **106,** 311 (1934).
21. Folin, O., and Denis, W., *J. Biol. Chem.*, **12,** 239 (1912).
22. Folin, O., and Macallum, A. B., *J. Biol. Chem.*, **11,** 265 (1912).
23. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83,** 103, 109 (1929).
24. Folin, O., and Trimble, H., *J. Biol. Chem.*, **60,** 473 (1924).
25. Folin, O., and Wu, H., *J. Biol. Chem.*, **38,** 82, 100 (1919).
26. Fujita, A., Iwatake, D., and Miyata, T., *Biochem. Z.*, **277,** 296 (1935).
27. Goudsmit, A., Jr., and Summerson, W. H., *J. Biol. Chem.*, **111,** 421 (1935).
28. Jackson, H., Jr., and Palmer, W. W., *J. Biol. Chem.*, **50,** 89 (1922); **53,** 373 (1922).
29. Lugg, J. W. H., *Biochem. J.*, **26,** 2144, 2160 (1932).
30. Medes, G., *Biochem. J.*, **29,** 2251 (1935).
31. Morris, J. L., and Macleod, A. G., *J. Biol. Chem.*, **50,** 55 (1922).
32. Roe, J. H., *Proc. Am. Soc. Biol. Chem.*, **8,** lxxv (1935); *J. Biol. Chem.*, **109** (1935).
33. Taylor, F. H. L., Chase, D., and Faulkner, J. M., *Biochem. J.*, **30,** 1119 (1936).
34. Wiener, R. v.E., and Wiener, H. J., *J. Lab. and Clin. Med.*, **11,** 1035 (1925-26).
35. Wu, H., *J. Biol. Chem.*, **43,** 189 (1920).

AMINO ACID CATABOLISM

IV. THE FATE OF CERTAIN SYNTHETIC α-AMINO ACIDS ADMINISTERED BY SUBCUTANEOUS INJECTION TO THE NORMAL DOG*

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(Received for publication, April 5, 1937)

We have studied the fate of a number of synthetic amino acids, in the normal dog maintained in nitrogen balance, to extend the knowledge of the mechanism of deamination of amino acids and of the influence of a methyl group on deamination and on catabolism in general. The purity of each of the compounds, which were obtained from the Eastman Kodak Company, has been checked by analysis. They were recrystallized as necessary.

Dogs have been maintained on a complete diet, prepared from purified materials (Cowgill), until the urinary nitrogen was reasonably constant. After the subcutaneous injection of a solution of an amino acid, evidence as to its fate is furnished by change or lack of change in the total nitrogen (Kjeldahl), urea and ammonia nitrogen (Van Slyke and Cullen), and amino acid nitrogen (Van Slyke and Kirk) of the urine. The return of the urinary values after the day of injection to the levels prevailing before strongly supports the view that the changes are attributable to the compound administered.

Two experiments have been performed with each amino acid except alanine. As the results have been in good agreement, only one of each pair of experiments is here presented (Table I). The

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* A report of this work was presented before the Thirtieth meeting of the American Society of Biological Chemists at Washington, March, 1936.

Based on a thesis submitted by John A. Leighty to the Faculty of Purdue University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy, June, 1936.

TABLE I

Disposal of Nitrogen of Subcutaneously Injected Amino Acids

Day	Total N	Urea N	Ammonia N	Amino acid N	Remarks
	gm.	*gm.*	*gm.*	*gm.*	
10	4.65	4.04	0.32	0.03	
11	4.74	4.09	0.33	0.02	
12	6.04	5.18	0.35	0.27	1.26 gm. N as *dl*-alanine
13	5.04	4.31	0.39	0.04	
14	4.85	4.20	0.32	0.04	
18	4.94	4.38	0.31	0.04	
19	5.23	4.62	0.35	0.04	
20	5.09	4.47	0.37	0.05	
21	5.90	5.12	0.45	0.12	0.80 gm. N as *dl*-2-aminobutanoic acid
22	5.23	4.63	0.33	0.03	
23	5.12	4.52	0.36	0.03	
17	5.70	4.94	0.37	0.03	
18	5.85	5.19	0.30	0.03	
19	5.61	4.89	0.29	0.03	
20	6.76	5.83	0.43	0.06	0.59 gm. N as *dl*-2-aminopentanoic acid
21	5.94	5.10	0.40	0.07	
22	5.78	5.04	0.29		
28	4.93	4.22	0.32		
29	5.00	4.27	0.33	0.04	
30	6.06	4.58	0.33	0.30	0.81 gm. N as 2-amino-2-methylpropanoic acid
31	5.28	4.43	0.37	0.05	
32	4.59	3.91	0.27		
33	4.90	4.22	0.31		
25	5.29	4.68	0.36	0.03	
26	5.28	4.61	0.38	0.02	
27	5.85	4.49	0.39	0.32	0.721 gm. N as *dl*-2-amino-2-methylbutanoic acid
28	5.34	4.39	0.56	0.07	
29	5.17	4.43	0.39	0.05	
30	5.31	4.57	0.38	0.05	
9	5.55	4.73	0.34	0.05	
10	5.67	4.86	0.34	0.05	
11	6.13	5.11	0.36	0.27	0.60 gm. N as *dl*-valine
12	5.68	4.94	0.30	0.03	
13	5.78	5.04	0.33	0.03	

extra total nitrogen accounting for the nitrogen of *dl*-alanine administered appeared as both extra urea nitrogen and extra amino nitrogen. It is to be concluded that most of the nitrogen of *dl*-alanine is split off to yield urea, but that some of the amino acid escaping attack is excreted. Abderhalden and Tetzner (1935) have found that after administration of the racemic mixture *d*(–)-alanine appeared in the urine. The nitrogen of *dl*-2-aminobutanoic acid was recovered largely as urea, with but a little extra amino nitrogen representing unchanged compound. *dl*-2-Aminopentanoic acid apparently stimulated nitrogen catabolism, but was itself practically completely degraded, as but little extra amino acid nitrogen appeared in the urine. Decrease of solubility with reduced tendency for rapid excretion may explain why increasing chain length was associated with a lower urinary excretion of unaltered compound. Friedmann (1908) fed *dl*-alanine, *dl*-2-aminobutanoic acid, and *dl*-2-aminopentanoic acid to dogs and from the N:C ratios of the urine concluded that these amino acids were completely utilized.

The presence of a methyl group on the carbon atom also holding the amino group rendered the latter resistant to removal by nitrous acid. In 22 minutes of contact with nitrous acid, *dl*-2-amino-2-methylbutanoic acid yielded nearly all, about 96 per cent, of its amino nitrogen, but 2-amino-2-methylpropanoic acid lost only about two-thirds as much in the same time. Values recorded for amino acid nitrogen of the urines containing these compounds have been obtained after exposure of 4 minutes to nitrous acid, and represent but a portion of the total present. Longer exposure has resulted in increasing contribution to the gas evolved of other substances in the urinary filtrate, making the blank high and rather inconstant. However, approximate estimates of amino acid nitrogen, with longer treatment with nitrous acid, have indicated practically quantitative recovery of these compounds in the urine after injection.

2-Amino-2-methylpropanoic acid was but incompletely attacked after injection. While there was some extra urea nitrogen, it probably originated from the tissue nitrogen lost, as the increase in total urinary nitrogen amounted to more than that in the compound administered. The extra amino acid nitrogen, as measured, accounted for approximately a third of that of the substance administered, but it is to be emphasized that the actual amount present was much more. In another experiment, after the injection of 5 gm. of this compound containing 680 mg. of nitrogen, by formol titration, 600 mg. of urinary amino acid nitrogen were found, and it was possible to isolate 4 gm. of unchanged material from the urine. Increased excretion of amino nitrogen, with absence of increased excretion of urea, is evidence that *dl*-2-amino-2-methylbutanoic acid escaped attack. Results with *dl*-valine may readily be explained to indicate that one optical isomer was catabolized but that one escaped attack. Unpublished experiments with the separate isomers (Corley and Snyder) confirm this.

SUMMARY

The following conclusions have been drawn for the compounds studied. In the normal dog, under experimental conditions employed, amino acids with straight chains yield their nitrogen as urea readily, those with a methyl group on the same carbon atom as the amino group lose their nitrogen with difficulty. A methyl group on the carbon atom adjacent to the one with the amino group may interfere with deamination. It is suggested that the latter effect is associated with spatial configuration.

BIBLIOGRAPHY

Abderhalden, E., and Tetzner, E., *Z. physiol. Chem.*, **232**, 79 (1935).
Friedmann, E., *Beitr. chem. Physiol. u. Path.*, **11**, 151 (1908).

MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

I. GASOMETRIC ANALYSIS*

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(Received for publication, May 8, 1937)

When a chloride solution is shaken with silver iodate powder, the reaction which takes place is

$$(1) \qquad \underset{\text{soluble}}{NaCl} + \underset{\text{insoluble}}{AgIO_3} \rightleftarrows \underset{\text{insoluble}}{AgCl} + \underset{\text{soluble}}{NaIO_3}$$

Although both AgCl and $AgIO_3$ are "insoluble" salts, the AgCl is so much the more insoluble that, under the conditions of this method, the reaction is almost quantitative from left to right; and it is completed within a minute. The iodate which goes into solution by the reaction serves as an accurate measure of the chloride present.[1]

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* A preliminary report of the work of this and Papers II and III (Sendroy, 1937, *a*, *b*) was given at Detroit, April, 1935, at the Meeting of the Federation of American Societies for Experimental Biology. This report (Sendroy, 1935) was apparently overlooked by Haslewood and King (1936) who have since its appearance published a paper entitled "A new iodometric procedure for the estimation of chloride in small amounts of blood." Their procedure is based, like the author's, on the use of silver iodate as a chloride precipitant, but their application is limited to Somogyi filtrates (1930) of serum and to titrimetric estimation of the iodate. The details of technique are quite different from those developed and preferred by the writer.

[1] Other "insoluble" silver salts, also more soluble than silver chloride, could be used in place of the iodate. Thus two, which the author has used with some degree of success before abandoning them in favor of the present procedure, are the chromate and the oxalate, for the reactions:

$$(a) \qquad 2NaCl + Ag_2CrO_4 \rightleftarrows \downarrow 2AgCl + Na_2CrO_4$$

This reaction offers several advantages for chloride analyses. The determination of the dissolved iodate affords a *direct* measure of the chloride, whereas measurements by difference, such as the Volhard titration used in most biological analyses, require two standard solutions and involve the cumulative errors of their measurements. These errors may be large when the concentration of chloride is relatively low. Furthermore, iodate is beautifully adapted to microanalyses, gasometric, titrimetric, or colorimetric. It is particularly suitable for the measurement of minute amounts of chloride, because in the oxidation-reduction reactions by which the iodate is measured, it functions as a hexavalent oxidizing agent. Thus 1 equivalent of chloride yields 6 equivalents of iodate to measure. These 6 equivalents are measured gasometrically by reaction with hydrazine as 1.5 moles of N_2; they are measured titrimetrically or colorimetrically by reaction with KI as 6 equivalents of free iodine. By the gasometric or titrimetric methods a precision of ±0.5 per cent can be obtained with as little as 0.1 cc. of serum. The colorimetric measurement is less exact.

The silver iodate procedure is applicable to salt solutions, to urine, to protein-free filtrates of plasma and of whole blood, and to plasma and serum without removal of proteins.

Of the three types of procedure for measuring the dissolved iodate, the writer prefers the gasometric, because of its speed, convenience, and freedom from the use of standard solutions. Fourteen or fifteen consecutive iodate determinations can be made gasometrically in an hour, with duplicates seldom differing by more than 1 part in 200.

In this paper the theoretical and experimental basis of the

(*b*) $2NaCl + Ag_2C_2O_4 \rightleftarrows \downarrow 2AgCl + Na_2C_2O_4$

The dissolved chromate or oxalate can be measured either gasometrically or titrimetrically. The chromate can also be measured colorimetrically, as in Isaac's (1922) colorimetric chloride method. For present purposes iodate, however, proved to be preferable. In what follows, it will be convenient to regard total halide (chloride, bromide, and iodide) simply as "chloride," since chloride is the predominant halide, especially in most biological material. However, under the conditions prescribed, $AgIO_3$ may be used for the determination of bromides and, in some cases, of iodides, as well as of chlorides.

reaction of chloride with silver iodate will be developed, and the gasometric method will be described in its macro, micro, and ultramicro forms. The titrimetric and colorimetric methods will be detailed in the accompanying papers.

In the gasometric method the iodate is measured in the Van Slyke-Neill (1924) manometric apparatus by the N_2 evolved instantaneously, according to the equation:

$$2NaIO_3 + 3N_2H_4 = \uparrow 3N_2 + 2NaI + 6H_2O \tag{2}$$

This reaction was introduced by Riegler (1902) for gasometric measurement of iodate, although not in connection with chloride analysis. The reaction was applied by Van Slyke, Hiller, and Berthelsen (1927), with the Van Slyke-Neill apparatus, to measure the iodate obtained in their gasometric methods for sulfate and total base.

The only gasometric chloride method previously described is that of Riegler (1901). He precipitated AgCl and caused the isolated precipitate to react with hydrazine in alkaline solution.

$$4AgCl + N_2H_4 + 4NaOH \rightarrow \downarrow 4Ag + \uparrow N_2 + 4NaCl + 4H_2O \tag{c}$$

For each mole of Cl 0.25 mole of N_2 was evolved. The present procedure yields 6 times as much N_2 and appears otherwise more practicable in detail than Riegler's, which did not attain application to biological material.

DESCRIPTION OF METHOD

Reagents—

Approximately 0.85 M Phosphoric Acid Solution—5.8 cc. of syrupy orthophosphoric acid, H_3PO_4 (specific gravity 1.7), of reagent grade are diluted to 100 cc. with water. *This solution must be tested for and found free of halides.*

Approximately 0.34 M, 0.17 M, and 0.085 M Phosphoric Acid Solutions—0.85 M solution is diluted 2.5, 5, and 10 times, respectively, with water.

M/15 Potassium Acid Phosphate Solution—9.08 gm. of KH_2PO_4, diluted to 1 liter with water.

Caprylic Alcohol—This is used to prevent foaming in biological samples.

Silver Iodate, Powder, C.P.[2]—This should be kept in an amber, glass-stoppered bottle and stored in a desiccator when not in use. Under these conditions, it keeps indefinitely.

Alkaline Hydrazine Solution—Equal volumes of saturated aqueous solution of hydrazine sulfate (Merck) and of 40 per cent (40 gm. per 100 cc. of solution) sodium hydroxide (Merck's Reagent) are mixed. *Before use, the freshly prepared alkaline hydrazine solution must be first cooled to room temperature, then saturated with air.* This may be accomplished by vigorous shaking with air for 30 minutes, or by allowing it to stand overnight in a loosely stoppered flask. Some alkaline hydrazine solutions used in this laboratory have shown marked signs of deterioration over periods of several weeks, yielding low results for iodate. As a general measure of precaution *no alkali-hydrazine mixture more than 21 days old should be used for gasometric iodate determinations.*

Tungstic acid reagent (*Folin and Wu, 1919*) is prepared as modified by Van Slyke and Hawkins (1928). 1 volume of 10 per cent sodium tungstate is mixed with 8 volumes of N/12 sulfuric acid. The solution must be freshly prepared every 2 weeks.

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[2] The silver iodate can be prepared as follows from two solutions: (1) *potassium iodate* (Merck's Reagent), 23 gm. in 600 cc. of water, and (2) *silver nitrate* (Merck's Reagent), 18 gm. in 400 cc. of water. To the iodate in a 2 or 3 liter beaker, the silver is added from a burette or dropping funnel, slowly, with constant stirring. After standing overnight in the dark, the precipitate is poured onto a Buchner funnel, and washed three times by decantation. It is then washed repeatedly on the filter with 100 cc. portions of water, to free it of excess iodate. Near the end of this washing process (about twelve washings) the 100 cc. portions of wash water are collected after each washing, and a 10 cc. sample taken into a test-tube. A few drops of 0.85 M H_3PO_4 and a few drops of 5 per cent potassium iodide are added to develop the yellow iodine color. Washing is continued until the yellow color becomes constant. The material is dried *in vacuo* at room temperature, overnight. It is then pulverized and stored in an amber, glass-stoppered bottle, and kept in a desiccator when not in use. The theoretical yield is about 30 gm. We have actually obtained 27 gm. of dried material, enough for from 270 to 4500 determinations. The purity of the material may be further tested (1) by a determination of its solubility in water, and (2) by its reaction with known chloride solutions. These determinations are discussed in the experimental sections. During these studies, silver iodate was not available on the market. Since then, Merck and Company, Inc., have prepared some according to the above directions. Their material, tested under a variety of conditions, has been found to be the same as that prepared by the writer.

Zinc hydroxide reagent (*Somogyi, 1930*), prepared as described by Peters and Van Slyke (1932).

Solution I—10 per cent zinc sulfate. 100 gm. of $ZnSO_4 \cdot 7H_2O$ in 1 liter of solution.

Solution II—0.5 N sodium hydroxide.

The two solutions must be so related that when 10 cc. of the zinc sulfate are titrated with sodium hydroxide, 10.8 to 11.2 cc. of the alkali are required to produce a permanent pink color with phenophthalein. The 10 cc. of solution are diluted with 50 to 70 cc. of water before the titration; the alkali is added slowly with continuous shaking.

Procedure

The general procedure consists of the following steps.

1. The sample is diluted in an acid solution (usually with 0.085 M phosphoric acid) to a pH of between 2.0 and 3.0, and to a chloride concentration of between 12 and 3 mM per liter.

2. With the addition of solid silver iodate and vigorous shaking, the chloride is precipitated and iodate is released into solution.

3. The silver chloride, together with excess silver iodate, is separated from the solution by centrifugation at high speed.

4. The supernatant liquid is then analyzed gasometrically for iodate.

5. A *blank* analysis, which serves for a whole series of gasometric measurements, is carried out.

The details of the steps involved are as follows:

1. Preliminary Preparation and Dilution of Sample for Analysis—The purpose of this treatment is to approximate, in the solution to be analyzed, concentrations of chloride and of hydrogen ions which have experimentally been found suitable for the chloride precipitation reaction. Dilution serves not only to facilitate the analysis by avoiding unnecessarily strong and impracticable concentrations of iodate, but also to decrease the concentrations of proteins and other possibly interfering substances, when present. The preliminary treatment and preparation will therefore vary somewhat with the nature and the available amounts of the material to be analyzed.

In general, a sample of any amount of original material, down to 0.02 cc., may be initially used. Depending on its chloride concentration, it is diluted with 0.085 M or 0.17 M phosphoric acid solution to a chloride concentration of between 3 and 12 mM. In any case, regardless of how much or how little dilution may be necessary to bring about these concentrations, as a rule (exceptions to which will be indicated further) the pH of the dilute solution should be brought to about 2.0 to 3.0 with phosphoric acid. The procedure for diluting various biological materials will be described in connection with their analyses.

Phosphoric acid has been adopted for general use in this method, because it has been found to answer the requirements of the proper conditions for serum analysis. In the analysis of urine samples slightly high results were sometimes obtained when the pH was over 3.5 or 4.0. Similarly, when M/15 KH_2PO_4, of pH 4.5, was used to dilute plasma or serum 10-fold, the average results were 2 per cent higher than when the chloride was determined by digestion methods. Furthermore, when serum was diluted more than 1:10 with M/15 KH_2PO_4, even before $AgIO_3$ was added, a precipitate appeared in the sample; probably serum albumin, the isoelectric point of which is approximately at pH 4.7. For both urine and serum a lower pH proved more satisfactory for the reaction of chloride with $AgIO_3$. Although acetic acid also had been used for urine, it was unsatisfactory for serum. A suitable acid, not reacting with silver, iodate, chloride, or protein, was found in orthophosphoric acid, H_3PO_4. It has therefore been used as a diluent for the procedures described in these papers.

In the development of the method we have usually carried out duplicates by precipitation of the chloride in two separate tubes, with one iodate analysis of the supernatant fluid from each. Very often each supernatant fluid was analyzed in duplicate. A significant deviation in the duplicate precipitation results has been so rare in our experience, that for *macro-* and *micro*procedures in the analysis of any of the materials mentioned we recommend the precipitation of but one sample, with duplicate analyses of the supernatant fluid.

2. Precipitation of Chloride with Silver Iodate—To the dilute, acidified solution silver iodate is added, about 10 mg. per cc. of solution being required. More does no harm; less is to be avoided. With little practise it is easy to estimate the necessary amount on the tip of a spatula. The tube is then covered by a rubber cap and shaken *vigorously* for 2 minutes by hand or in a shaking machine. We have utilized the manometric apparatus itself (Fig. 1) for this purpose by wrapping a towel or cloth around the jacket of the extraction chamber and fastening the tubes together with intertwining rubber bands. The tubes should all be with their axes in a horizontal position and pointing in the same direction as the driving shaft which shakes the chamber. The reaction with silver iodate is practically instantaneous but shaking is continued to saturate the solution exactly with air, which subsequently is liberated in the gasometric determination, together with the N_2 freed by action of the iodate on N_2H_4. To make sure that the air included shall be the same as in the blank analysis, it is important to have the solutions at room temperature before the analyses are begun.

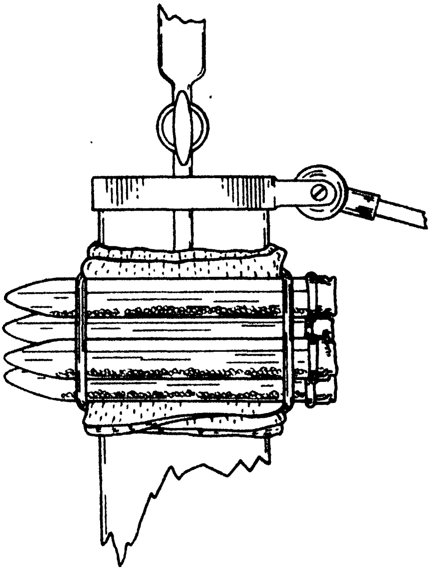

FIG. 1. Centrifuge tubes attached to outer jacket of the extraction chamber, for shaking with solid $AgIO_3$.

3. Separation of Solid Silver Salts from the Supernatant Fluid—After the shaking, subsequent sampling may be facilitated, if the caps are removed and the necks of the tubes are wiped free of precipitate. For small volumes, as in the *ultramicro* serum analysis, this is unnecessary. The tubes are covered again with clean caps and centrifuged for 1 minute[3] at a speed greater than 3000 R.P.M. The resulting supernatant liquid, which is almost completely clear, is then analyzed for iodate.

4. Analysis of Liberated Iodate in the Supernatant Fluid—Interfering substances in some materials may reduce acid solutions of iodate at an appreciable rate. Such is the case in the analysis of certain types of urines. For such material the iodate analysis should be made within 1 hour after the centrifugation. Serum samples may stand for at least 3 hours before analysis. Salt solutions and protein-free filtrates may stand for more than 24 hours without change. The number of samples simultaneously shaken with silver iodate, therefore, depends on the length of time which may be allowed to elapse between the first and last iodate determinations of the several supernatant samples.

When samples are being taken for analysis from the centrifuged tubes, care should be exercised in avoiding their contamination with solid silver iodate or chloride, from either the surface or the bottom of the centrifuged liquid. Usually it will suffice to close the stop-cock of the pipette, and then, without touching the sides of the tube, to plunge the tip of the pipette quickly below the surface. If the tip is held no nearer than ½ inch from the precipitate, the sample may be withdrawn, without contamination, by gentle suction controlled by the pipette stop-cock.

However, contamination may easily be avoided with certainty by a cotton filter device similar to that of Van Slyke and Hawkins (1928). A little cotton is twisted to a point, then sucked by vacuum into the tip of the sampling pipette. The protruding cotton is cut off at about 2 mm. from the tip of the pipette which is then plunged below the surface for withdrawal of the sample.

[3] For salt solutions, normal urines, and protein-free filtrates, filtration through halide-free filter paper ("ashless" filter paper washed free of halide in distilled water and then dried) may be carried out to separate the silver salts from the supernatant liquid. When protein is present, however, highest accuracy is obtained only by centrifugation. Filtration of protein-containing material (serum or urine) gives less consistent and higher results (by about 1 per cent) for both the gasometric and titrimetric (Sendroy, 1937, *a*) measurements. Moreover, loss of fluid on the filter paper eliminates filtration as a practical procedure in the analysis of small volumes of supernatant fluid.

Before accurate adjustment to the upper mark of the pipette the cotton is removed. In handling small volumes of supernatant fluid, as in the *ultramicro* serum analysis, this cotton plug is a *necessary* device. In these cases the need of obtaining most of the supernatant fluid for analysis makes it necessary to hold the tip of the sample pipette so close to the centrifuged solid material that contamination would be inevitable without the protection of the cotton filter.

Gasometric Determination of Total Dissolved Iodate—As found by Van Slyke, Hiller, and Berthelsen (1927), the reaction between hydrazine and iodate is quantitative. The author has confirmed their results, but has obtained consistently accurate results only when the indicated precautions for the use of the hydrazine reagent (see "Reagents") and for the technique of iodate measurement given below were observed. Under these conditions the nitrogen yields were 100.0 ± 0.3 per cent of theoretical for dozens of known potassium iodate and biiodate analyses covering a wide range of concentrations. The results were the same whether the iodate was dissolved in water or in 0.085 M H_3PO_4 solution.

The manometric apparatus (Van Slyke and Neill, 1924; Van Slyke, 1927) must be kept scrupulously clean, and, if possible, should be reserved only for iodate determinations. If used for analyses involving other substances which may react with hydrazine or iodate, suitable precautions should be taken against contamination from such sources (*e.g.* see p. 413, Peters and Van Slyke (1932)). It is well to change most of the mercury in the system once a month, simply by evacuating the chamber as for extractions, closing the stop-cock leading to the leveling bulb, and pouring off as much of the mercury from the leveling bulb as is possible without allowing air into the rubber tubing. Fresh mercury is then used to refill the leveling bulb.

Before the first analysis of a series, the apparatus is freed of air and of any substance which might oxidize hydrazine to N_2 gas. This is done by mixing 2 cc. of the alkaline hydrazine reagent with 2 cc. of distilled water in the extraction chamber, evacuating the chamber, and shaking for 1½ minutes. The evolved gas and solution are then expelled. 10 or 15 seconds are allowed for solution adherent to the inner wall of the chamber to rise to the top above the mercury. This solution is then also expelled.

The analysis is begun by placing about 3 cc. of the alkaline hydrazine solution in the cup of the chamber. With the leveling bulb on a level with the bottom of the chamber, and its control stop-cock open, hydrazine solution is admitted into the chamber until it *exactly* reaches the 2.0 cc. mark. About 1 cc. of mercury is poured into the cup, a little is allowed to run into the chamber, and the excess hydrazine above the mercury is removed with two washings of water. Samples of the centrifuged supernatant fluid are admitted into the chamber through the mercury seal, covered with a layer of water, as described by Van Slyke (1927). (See also Peters and Van Slyke (1932) Fig. 52, p. 344.) A 1 or 2 cc. stop-cock pipette is used.[4] The leveling bulb of the apparatus is kept at the same level as for the admission of the hydrazine, and the admission of the sample is controlled by the *stop-cock at the top of the extraction chamber.* The reaction between iodate and hydrazine is so rapid that some of the evolved N_2 gas would escape up through the pipette were the sample not delivered at slight negative pressure. The pipette is slowly lifted up out of the cup, and almost all of the mercury in the cup is allowed to enter the chamber to form a seal through the stop-cock.[5]

The sample having been introduced, it is *first thoroughly mixed with the hydrazine reagent in the chamber*, by slightly raising and lowering the leveling bulb of the apparatus, and by shaking the chamber several times by hand. The chamber is then evacuated so that the mercury surface lies near the 50 cc. volume mark, about 1 cm. below the curve of the bulb. After vigorous shaking for 1½ minutes,[6] the pressure of the gas evolved is read at the 0.5 or 2.0 cc. volume, and is p_1 in the calculation. The temperature is recorded.

5. The Blank—A gasometric analysis on an equal volume of either another aliquot portion of the diluted sample or of the diluent alone is carried out simultaneously with that on the sample through steps (1), (2), (3), and (4) of the procedure outlined, exactly as above, *except that no silver iodate is used.* The result of the blank determination gives the base-line reading p_0 for all analyses, and serves to correct for the amount of air dissolved in the hydrazine reagent and in the supernatant sample analyzed. In running a series of determinations, it is well to make one gasometric measurement on a portion of the blank solution once in the beginning and another at the end of the series.

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[4] For the comparatively rare instances when less than 1 cc. of supernatant fluid is available for the gas analysis, see the detailed directions for the *ultramicro*analysis of serum, given in the following (p. 351).

[5] The error involved in the transfer of samples through a mercury seal is discussed in the section on "Calculations."

[6] Upon being first shaken, some mixtures containing biological material foam in spite of the caprylic alcohol. In such cases, the shaking should be stopped and the foam allowed to subside. Upon resumption of the shaking, no further foaming will occur.

In most of the analyses to be described, the original sample material is diluted to such an extent that it has practically no effect on the amount of air contained in the supernatant fluid used for analysis. It is therefore not necessary to use any of the original sample material for a blank analysis. Instead, the blank determination may be made on the diluent used for preparing the original material for analysis. Thus, in the analysis of serum without deproteinization, or of highly diluted urine, the blanks are made on the 0.085 M or 0.17 M H_3PO_4 solutions used for dilution. In the analysis of protein-free filtrates, the blanks are made on a mixture of the same reagents used to precipitate the proteins in the original sample, with water used in place of the serum or whole blood. *In all cases the same solution or solutions must be used for the blank that are used for the dilution of the original sample,* in order that the air content correction may truly represent that of the diluted supernatant sample analyzed for iodate. The volumes of the solutions shaken in the centrifuge tubes should be approximately the same for both the blank and the supernatant fluid analyzed. Also, the temperature and length of time of shaking, with and without $AgIO_3$, for both sample and blank, respectively, must be the same, otherwise the blank may not provide an accurate correction.[7]

Since the apparatus is automatically rendered air-free with each analysis, *no washing of the apparatus is required between analyses* of either samples or blanks. Analyses of the centrifuged iodate solutions can, therefore, be made in rapid succession, at the rate of fourteen or fifteen an hour.

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[7] The author has investigated experimentally, the possibility of eliminating the actual determination of gasometric blank readings altogether, first, by *calculating* the air content of the samples from their temperature and the barometric reading, and secondly, by making the sample air-free before analysis. It was found that the actual determination of p_0 from the blank, as described in the text, was simpler, less time-consuming, and more accurate.

Application of the above general procedure to different types of material will now be detailed.

A. Determination of Chloride in Inorganic Material—Samples are diluted with a suitable phosphoric acid solution to a final chloride concentration of 3 to 12 mM per liter, and a pH of about 2.0 to 3.0. The phosphoric acid concentration of the diluted solution should be no more than 0.17 M. Thus, approximately 0.1 M neutral salt solutions, or 0.1 N HCl solutions, would be diluted 10 to 30 times with 0.085 M H_3PO_4, then analyzed exactly as described for serum analyses below, except that no caprylic alcohol is necessary.

Solutions containing too much free acid or alkali to give the above pH when diluted with 0.085 M or 0.17 M H_3PO_4 should first be neutralized to alizarin red with sodium hydroxide (*chloride-free*) or phosphoric acid. The neutralized material is then diluted further by the addition of 0.085 M phosphoric acid. For solutions containing iodide the final pH should be somewhat higher (4.5), and dilution should be made with M/15 potassium acid phosphate. *Solutions used for dilution should be chloride-free when tested with silver nitrate and nitric acid.*

B. Determination of Chloride in Urine—The presence of albumin or blood in the urine does not prevent the application of the gasometric chloride method to the analysis of such material. A slight effect of the albumin is corrected for in the calculations. All samples (preferably obtained without preservative)[8] are diluted with 0.17 M H_3PO_4, which strength of the acid is sufficient to bring almost any sample of urine, in the dilutions employed, to a pH of 3.5 or lower. The method of diluting samples will depend on whether speed and convenience, or whether accuracy is the primary consideration.

In the following, two procedures for urine analysis will be described: (*a*) a routine rapid method applicable to all samples, and

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[8] If the use of a preservative should be necessary, it is recommended that a few crystals of thymol (Peters and Van Slyke, 1932) be used. Toluene should be avoided, as its use in any gasometric method is very likely to cause difficulties on account of its vapor tension and its air solubility.

(*b*) a more refined technique of individual treatment of samples, for maximum accuracy.

Routine Urine Method—A 0.5 cc. sample of urine is pipetted into a graduated 15 cc. conical Pyrex centrifuge tube. A drop of caprylic alcohol is added, then 0.17 M H_3PO_4 to the 10 cc. volume mark. About 0.1 gm. of $AgIO_3$ is added to the tube, which is then capped, shaken for 2 minutes, centrifuged,[9] and analyzed as described in the general procedure (steps (2), (3), and (4) above).

Samples of 1 cc. or 2 cc. of the supernatant fluid are withdrawn and analyzed gasometrically within 1 hour after centrifugation. The pressure readings are made at the 0.5 cc. volume when p_1 is not more than about 560 mm.; otherwise, when more N_2 is present, they are read at the 2.0 cc. volume. The *blank* analysis for the p_0 reading is carried out with the *same* 0.17 M H_3PO_4 solution used to dilute the urine. A 10 cc. portion is shaken at the same time, without the addition of $AgIO_3$. Samples of 1 cc. or 2 cc. are analyzed gasometrically as above.

With this technique the accuracy of the analyses of urine samples of chloride concentration between 60 mM and 250 mM per liter (3.5 to 14.6 gm. of NaCl per liter) is limited only by the accuracy with which one may dilute to volume in a graduated centrifuge tube. The tubes used for this purpose should have the 10 cc. volume mark checked by delivery of water from an accurate 10 cc. pipette. When the agreement is within 0.1 cc., an accuracy of 1 per cent may be expected of these analyses.

In the case of the relatively few urine samples of more than 250 mM of chloride per liter, the required excess of $AgIO_3$ may not be sufficient, and greater dilution than 20 times may, therefore, be necessary. In the case of samples below 60 mM in chloride, the 20-fold dilution gives supernatant samples of less than 3 mM. Under these conditions, the possible errors are increased, but an accuracy of 5 per cent or better may be obtained, which is sufficient for most clinical purposes. The errors are minimized by a correction in the calculations for the solubility of $AgIO_3$ in the supernatant samples analyzed, and by the use of 2 cc. samples of supernatant fluid with the nitrogen measured at the 0.5 cc. volume, so that maximum pressure readings may be obtained.

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[9] See foot-note 3. Most samples of urine may be filtered if centrifugation happens to be inconvenient. However, when protein is present, centrifugation is necessary to avoid errors which are further increased, if at the same time the chloride concentration is low.

In some pathological conditions the application of the *routine method* to urines with low chloride content may result in errors greater than 5 per cent. Such cases are discussed in a following paper (Sendroy, 1937, *c*). Under those conditions, and in any case in which an accuracy of better than 5 per cent must be obtained, the following *precise method should be used.*

Precise Urine Method—When it is desirable that the maximum accuracy (±0.5 per cent) be obtained for any given urine sample, the dilution with 0.17 M H_3PO_4 is so made that the diluted sample may be between 3 mM and 12 mM, preferably about 8 mM, in chloride concentration. The rest of the determination is then carried out exactly as described above. Samples of 2 cc. of the supernatant fluid are analyzed, and pressure readings are made at the 2.0 cc. volume.

Under these conditions, the extent of dilution will vary from one sample to another, depending on its chloride concentration. A preliminary analysis by the 20-fold dilution method of the above *routine procedure* may be made to determine this probable concentration, and hence the optimum dilution. There is, however, an easier procedure. An approximate indicator of the necessary dilution of any given urine sample is furnished by the reading of the specific gravity (to two decimal places, uncorrected) which, from Sendroy, Seelig, and Van Slyke's results (1934) and from our own, varies with the total base and chloride in an approximately linear manner. Chloride-poor urines obtained from patients on low salt diets also have a linear relationship, but one which is quantitatively different from that of normal urines. Such samples require relatively less dilution. Thus, the dilution for any sample will be determined by its specific gravity, as indicated in Table I. Samples of 1 cc. are diluted in volumetric flasks to the final volume indicated.[10] For dilution to 3 times the volume, 5 cc. of urine are diluted in a 15 cc. flask, or 2 volumes of 0.17 M H_3PO_4 are added to 1 volume of urine. By these dilutions the chloride will be within the desirable limits of concentration of 3 and 12 mM in all but a very few cases.

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[10] Volumetric flasks of 5 cc., 20 cc., or 30 cc. capacity are not carried in stock as standard equipment in supply houses in this country. They may easily be obtained, on special order, from several of the more prominent glass-blowing firms. We have imported ours directly from Gebruder Schubel, Frauenwald i. Thur., or Fettke & Co., Döbern, N.-L., Germany.

The gasometric analysis of 2 cc. of the supernatant fluid is carried out as described above for the *routine procedure.* The *blank* analysis is likewise made on the same 0.17 M H_3PO_4 used to dilute the urine. In the rare cases in which the maximum accuracy may be required in the analysis of chloride-poor samples, if the dilution used is 1:3, the blank analysis should be made on another portion of approximately the same volume of diluted urine, treated in the same way as is the one analyzed for chloride, except that no $AgIO_3$ is used.

TABLE I

Dilutions of Urine Samples According to Specific Gravity, for Analysis by the Precise Method

Specific gravity (uncorrected) of sample of urine	Dilution with 0.17 M H_3PO_4 of 1 volume of urine to multiple of the volume indicated	
	Normal urine	Low chloride urine
1.005	5	3
1.010	10	
1.015	15	
1.020	20	5
1.025	25	
1.030	30	10

C. Determination of Chloride in Plasma or Serum, without Deproteinization—Samples, which may be from 1.00 cc. to as little as 0.02 cc., are diluted with 0.085 M phosphoric acid to from 10 to 31 times the original volume. The larger samples (1.0 and 0.5 cc.) are easier to pipette accurately. On the other hand, scarcity of material, as in pediatric work or in experiments on small animals, may make desirable the use of 0.02 to 0.2 cc. Indeed, the author has analyzed samples of 0.02 cc. gasometrically and titrimetrically with an accuracy closely approximating the results obtained with larger amounts of material (Table XXI). A nitrogen pressure of about 90 mm. is available for measurement in the gasometric analysis of 0.02 cc. samples. In what follows, for the sake of convenience, the procedure will be classified as *macro*, *micro*, or *ultramicro*, depending upon the size of the sample used, 0.5 cc., 0.1 cc., or 0.02 cc. The same procedures are applicable to non-protein-containing fluids, the chloride concentration of which approximates 0.1 M.

Macro Plasma Analysis—A sample of 0.5 cc. of serum or plasma is delivered from an Ostwald pipette, calibrated between marks, into a 10 cc. volumetric flask. 1 drop of caprylic alcohol is added. The sample is then diluted to the mark with 0.085 M phosphoric acid. As a general procedure for plasma or serum, the macroprocedure with 0.5 cc. samples will be found satisfactory from the standpoint of convenience (only one pipetting being required for the dilution) and sufficiency of supernatant sample for the iodate analysis.

The diluted sample is transferred to a 15 cc. Pyrex centrifuge tube, 0.1 gm. of $AgIO_3$ is added from the tip of a spatula, and the tube is covered with a rubber cap. It is then shaken for 2 minutes, centrifuged for 1 minute, and analyzed gasometrically, as described in detail in the general procedure in sections (2), (3), and (4) above.

Samples of 1.0 cc. of the supernatant solution are taken into a 1 cc. stop-cock pipette usually without a cotton plug.[11] The pressure readings p_1 for the gasometric analyses are taken at the 0.5 cc. volume.

Blank—In another centrifuge tube 10 cc. of the same 0.085 M H_3PO_4 used for the dilution of the serum are shaken *without* $AgIO_3$ and centrifuged simultaneously with the dilute serum + $AgIO_3$. A 1 cc. sample is used for the gasometric analysis, and the pressure reading, p_0, is taken at the 0.5 cc. volume. If a series of gasometric analyses requiring over an hour is run, duplicates of the blank are made on two 1 cc. aliquots of the same solution, one at the beginning and the other at the end of the series.

Micro Plasma Analysis—Samples of 0.2 cc. or 0.1 cc. of plasma or serum are delivered from pipettes calibrated *to contain* into 15 cc. Pyrex centrifuge tubes into which there have previously been accurately pipetted 5 or 3 cc., respectively, of 0.085 M H_3PO_4. In the former case, a *small* drop of caprylic alcohol (0.01 cc.) is added, while in the latter case, the end of a wire dipped in caprylic alcohol is immersed in the solution. For the 0.2 cc. and 0.1 cc. analyses about 50 mg. and 30 mg. of $AgIO_3$ are added, respectively. The rest of the analysis is carried out in detail exactly as for the *macro*determination. Samples of 1 cc. of the supernatant fluid are taken with a stop-cock pipette equipped with a cotton plug. The *blank* analysis is carried out as above, without $AgIO_3$, with 5 cc., or 3 cc. of the 0.085 M H_3PO_4 used for the serum dilution.

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[11] In the analysis of plasma or serum, a thin layer of fat-like substance mixed with caprylic alcohol may sometimes be present on the surface of the centrifuged solution. This should be dispersed throughout the liquid by gentle stirring before samples are withdrawn into a pipette equipped with a plug of cotton, as described in section (4) above.

Ultramicro Plasma Analysis—Samples of 0.05 cc. or 0.02 cc. are delivered from pipettes calibrated *to contain*, into small Pyrex test-tubes (13 × 100 mm.) into which there has previously been accurately pipetted 1.5 cc. or 0.6 cc., respectively, of 0.085 M H_3PO_4. A very *thin* wire dipped into caprylic alcohol and briskly shaken free of adhering fluid is touched to the surface of the solutions. For the 0.05 cc. and 0.02 cc. analyses, about 15 mg. and 6 mg. of $AgIO_3$ are added, respectively. *For the 0.05 cc. analysis*, the rest of the procedure is carried out in detail exactly as for the macrodetermination. Samples of 1 cc. of the supernatant fluid are taken with a stop-cock pipette equipped with a cotton plug. The *blank* analysis is carried out with 1.5 cc. of the same 0.085 M H_3PO_4 used for the serum dilution.

For the 0.02 cc. analysis, the procedure is similar, but with a slight difference in the gasometric analysis of the supernatant fluid. The small volume of supernatant fluid in this case (0.62 cc.) makes necessary the use of a 0.5 cc. pipette *without a stop-cock*, calibrated between marks, the lower mark being near the tip. A cotton plug filter is used in the pipette. After admission of the alkaline hydrazine to the manometric chamber the cup is washed thoroughly with water, the sample of supernatant fluid is pipetted into the cup, then likewise admitted into the extraction chamber. The lower part of the cup is washed with 1.0 cc. of water measured from a pipette, and this also is allowed to run into the chamber before the mercury seal is made. The volume of wash water should be within 0.01 cc. the same as in the blank analysis, because the dissolved air would affect the results if the variation were greater. The *blank* analysis is carried out exactly as above with 0.6 cc. of the same 0.085 M H_3PO_4 used for the serum dilution.

Measurement of Ultramicro and Micro Samples—The point in the *ultramicro-* and also in the *micro*method, where accuracy demands especial precaution, is not in the subsequent measurement of the iodate, but in the measurement of the small samples of serum or other sample material in pipettes calibrated *to contain.* The precautions add little to the time required, but the procedure is not a casual one and requires a little practise before good results are obtained. The major difficulty seems to lie not in the calibration or of the reading of the volume of the pipette, but in securing a volume of sample corresponding to that of mercury held between the tip and the volume mark when the pipette is calibrated. Discrepancies in this respect are usually the result of inadequate wiping of the tip. As a result, there may be traces of sample adhering to the outside around the tip. If the tip itself is actually touched with the wiping material, cloth, or filter paper, there may be an imperceptible loss at that point, because capillary attraction may withdraw the fluid a little from the pipette tip to the wiping material. On the other hand, if the tip is not wiped well enough, there may be an imperceptible protuberance of solution from the pipette, and an increased volume of sample. The author has adopted a simple, rapid technique for pipetting such small volumes with accuracy. Adherence to the following precautions has been found absolutely necessary.

In the first place, the pipettes used must be scrupulously clean and free flowing. They should be kept in chromic acid cleaning mixture when not in use. Before a pipette is used, it is thoroughly rinsed with distilled water, then with redistilled acetone (Commercial Solvent, or C.P. grade), and is dried in a few seconds with an air current. The sample to be analyzed is drawn up into the pipette only about 4 mm. above the calibration mark. The outside of the pipette, within several cm. of the tip, is then flushed with a thin stream of distilled water, after which the tip is slightly immersed in a small beaker containing about 50 cc. of water. The sample solution is then allowed to run down *slowly* into the water, until the meniscus reaches the mark. The tip is at once lifted from the water and is carefully wiped free of water with halide-free filter paper. The tip and the meniscus are again inspected to make certain there has been no additional drainage owing to capillary action. If the inspection is satisfactory, the pipette is immersed in the previously measured portion of phosphoric acid, and washed up and down five or six times. In the washing process the solution should not be drawn more than about 2 mm. above the pipette calibration mark.

In the *ultramicro*procedure, measurement of the sample and inspection of the tip are best made with the aid of a magnifying lens clamped to a ring-stand. The ultramicropipette is made of capillary tubing of about 0.5 mm. bore. A length of about 23 cm. and of 9 cm. from the tip corresponds to a volume of 0.05 cc. and 0.02 cc., respectively. For the calibration of micropipettes see Peters and Van Slyke ((1932) p. 18).

D. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—Deproteinization of plasma or serum is unnecessary for the gasometric or titrimetric analysis. Techniques for use with the tungstic acid filtrate of Folin and Wu (1919) and the zinc hydroxide filtrate of Somogyi (1930) will be described, however, because, when these filtrates are prepared for other analyses, it may be convenient to use them for chloride also. *Removal of proteins from whole blood is necessary.* For blood analysis, tungstic acid filtrates are recommended.

(*a*) *Tungstic acid filtrates of plasma or serum* may be used, prepared according to Folin and Wu (1919) or according to the modification of Van Slyke and Hawkins (1928) who mixed the sulfuric acid and sodium tungstate before use. In these filtrates the plasma is diluted to 10 times volume. Caprylic alcohol is added before deproteinization.

As much of the filtrate as is available, up to 10 cc., is transferred to a 15 cc. Pyrex centrifuge tube and $AgIO_3$ is added, 10 mg. per cc. of filtrate. The rest of the analysis is carried out as described above under "Procedure" (sections (2), (3), (4), and (5)).

Samples of 1 or 2 cc. of the supernatant fluid may be taken for gasometric iodate determination with the N_2 pressure measured at 2.0 cc. volume. If there is a scarcity of filtrate, samples of 0.5 cc. of the supernatant fluid taken in a pipette without a stop-cock may be used, and the N_2 pressure measured at 0.5 cc. volume, as described above for the ultramicroanalysis without deproteinization of 0.02 cc. of serum.

A *blank* analysis is made on another portion of tungstic acid filtrate treated in every detail as the one analyzed for chloride, except that no $AgIO_3$ is used. If the amount of available filtrate is insufficient, the blank may be carried out on an approximately equal volume of a mixture of the same tungstic acid reagents, with water used in place of plasma or serum.

(*b*) *Tungstic acid filtrates of whole blood* may be used, *but not the usual Folin-Wu filtrate.* The dilution of the sample is to 25 times the volume, and a change in the addition of the reagents is required.

Macrodetermination—In a 25 cc. volumetric flask, a 1 cc. sample of blood, about 3 cc. of water, and 1 drop of caprylic alcohol are placed. There are then added about 15 cc. of the mixed Van Slyke-Hawkins reagent, slowly, with stirring. Finally, 0.34 M H_3PO_4 is added to the volume mark. The flask is vigorously shaken, allowed to stand ½ hour, and the filtrate separated from the precipitated protein[12] by centrifugation. 10 cc. of the filtrate are treated with $AgIO_3$, as in the case of serum filtrates.

Microdetermination—To a 0.2 cc. sample of blood in a 5 cc. measuring flask are added 0.5 cc. of water, about 0.01 cc. of caprylic alcohol, 3.0 cc. of tungstic acid reagent, and 0.34 M H_3PO_4 to the mark. The filtrate is separated from the precipitated protein, as above. As much of the filtrate as is available is treated with $AgIO_3$.

Analysis of these blood filtrates is carried out as described above for serum filtrates. Samples of 1.0 cc. of the supernatant fluid are analyzed, with N_2 pressure measured at the 0.5 cc. volume. Blank analyses are made on equal volumes of filtrate, or reagents, as in serum filtrate analyses.

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[12] When enough sample (1 cc. of blood) is available, filtrates may be separated from the precipitated protein by filtration through ashless filter paper previously washed free of chloride in distilled water and dried. However, more "filtrate" is secured by centrifugation, which method alone can be used in the deproteinization of small samples. For convenience we shall refer to the centrifuged solution after addition of $AgIO_3$, as "supernatant" fluid, to the protein-free solution, however, obtained after addition of a protein precipitant, as "filtrate."

(*c*) *Zinc hydroxide filtrates of plasma or serum* may be used, prepared as usual, by dilution to 10 times the volume, with the reagents of Somogyi (1930), as described by Peters and Van Slyke ((1932) p. 481). The filtrate is further prepared for analysis by the accurate addition of 1 volume of 0.85 M H_3PO_4 solution to 10 volumes of filtrate.[13] The procedure from this point on is the same as that described for the analysis of tungstic acid filtrates of serum described above. Depending upon the amount of supernatant fluid available, gasometric determinations are made with 1.0 or 2.0 cc. samples with gas measurements at 2.0 cc. volume, or with 0.5 cc. samples with gas measurements at 0.5 cc. volume. *Blank* analyses are carried out with an approximately equal volume of another portion of filtrate.

If the filtrate available should be insufficient, the blank may be carried out just as well on approximately an equal volume of the same mixture of Somogyi reagents and 0.85 M H_3PO_4 with water used in place of the serum.

Calculations

The pressure, P, of N_2 evolved by reaction of iodate and hydrazine is $P = p_1 - p_0$. p_1 is the manometer reading obtained in the iodate determination of the supernatant fluid of the chloride precipitation. p_0 is the reading observed in a blank analysis at the *same temperature* as the p_1 reading. The chloride concentration is obtained by multiplying P by the proper factor from Tables III, IV, and V.

Temperature Correction for p_0—When the temperature changes between the p_1 and p_0 readings, it is not necessary to carry out another blank determination in order to obtain the correct p_0. One merely corrects the observed p_0 to the temperature of the p_1 reading by adding or subtracting the correction indicated in Table II. The p_0 reading is a function of the air contained in the sample and reagents, and extracted in the chamber during the analysis, and of the vapor tension of the mixed solutions. At any given temperature, the vapor tension will be fixed at a definite value, but the air content will vary with the volume S of the extracted solutions. A change in temperature will cause a change in both components of the p_0 reading. The corrections to p_0 in Table II are, therefore, given as a function of temperature change and of the total volume S of the extracted reagents in three ranges of temperature.

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[13] For routine work it may be found more convenient to put 1 volume of 0.85 M H_3PO_4 solution in a volumetric flask of 10 volumes capacity, and to dilute to the mark with the filtrate.

Correction for Loss of Sample in Transfer Through a Mercury Seal—Van Slyke (1927) has called attention to the error involved in the transfer of solution from a pipette to the chamber, through a mercury seal, without washing. He found a loss of from 0.005 cc. to 0.007 cc. of sample when the pipette was withdrawn from the cup. He, accordingly, recommended that the mercury seal be used only for samples of 2 cc. or greater volume.

TABLE II

Temperature Corrections for p_0

When the temperature of the p_1 reading is *higher* than that of the blank p_0 reading, the correction is *added* to the observed p_0; when p_1 temperature is *below* p_0 temperature, the correction is *subtracted* from p_0. Δt is the temperature change between the p_1 and p_0 readings. S is the total volume of solution extracted during the analysis, and includes 2 cc. of alkaline hydrazine solution in each case, the sample, and wash water when used. a is the volume mark of the extraction chamber at which p_1 and p_0 are read.

Δt		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	Range of temperature readings
S	a	Corrections to p_0										
cc.	cc.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	°C.
3-6	2.0	0.1	0.2	0.2	0.3	0.4	0.5	0.6	0.6	0.7	0.8	16-20
3-4	0.5	0.1	0.2	0.3	0.4	0.5	0.5	0.6	0.7	0.8	0.9	
4.5-6	0.5	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.1	
3-6	2.0	0.1	0.2	0.4	0.5	0.6	0.8	0.9	1.0	1.2	1.3	21-25
3-4	0.5	0.1	0.3	0.4	0.6	0.7	0.8	1.0	1.1	1.2	1.4	
4.5-6	0.5	0.2	0.3	0.5	0.6	0.8	0.9	1.1	1.2	1.4	1.6	
3-6	2.0	0.2	0.3	0.5	0.7	0.8	1.0	1.2	1.4	1.5	1.7	26-30
3-4	0.5	0.2	0.4	0.5	0.7	0.9	1.1	1.3	1.4	1.6	1.8	
4.5-6	0.5	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	

In the use of 1 cc. samples, as in the writer's serum method, the error resulting from such loss of sample would be significant. To avoid this error, a technique of transfer with washing, such as that

TABLE III

Factors for Calculations, from Observed P_{N_2} Measurements, of Precise and Routine Gasometric Urine Chloride Analyses

Temperature	For precise method; factor F for calculating according to Equation 5*	For routine method; dilution = 20, supernatant sample = 2.0 cc.†							
		Normal urine, K = 0.998				Albuminous urine, K = 0.990			
		a = 0.5		a = 2.0		a = 0.5		a = 2.0	
		Factor by which P_{N_2} is multiplied to find							
		mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter
°*C.*									
15	0.03710	0.1862	0.01089	0.745	0.0436	0.1877	0.01098	0.751	0.0439
16	0.03696	55	85	42	34	70	94	48	38
17	82	48	81	39	32	63	90	45	36
18	68	41	77	37	31	56	86	43	35
19	55	35	73	34	29	50	82	40	33
20	0.03642	0.1828	0.01069	0.731	0.0428	0.1843	0.01078	0.737	0.0431
21	29	22	65	29	26	37	74	35	30
22	16	15	62	26	25	30	71	32	28
23	03	09	58	23	23	24	67	29	26
24	0.03590	02	54	21	22	17	63	27	25
25	0.03578	0.1796	0.01051	0.718	0.0420	0.1811	0.01059	0.724	0.0424
26	66	90	47	16	19	05	55	22	22
27	54	84	44	14	18	0.1799	52	20	21
28	42	78	40	11	16	93	48	17	19
29	30	72	37	09	15	87	45	15	18
30	0.03518	0.1766	0.01033	0.706	0.0413	0.1781	0.01042	0.712	0.0417
31	06	60	30	04	12	75	39	10	15
32	0.03493	53	26	01	10	68	34	07	14
33	81	47	22	0.699	09	62	31	05	12
34	69	41	18	96	07	56	27	02	11

[*] For the precise method, under conditions of analysis other than those given above, calculation is made according to Equation 5 directly.

[†] If supernatant samples of 1.0 cc. are used, results are multiplied by 2. The factors for the routine method have been derived from Equation 5, as described on p. 363 for the conditions indicated above. If urine analyzed by the routine method is calculated to contain less than 60 mM of Cl per liter (or 3.51 gm. of NaCl per liter) correction for $AgIO_3$ solubility must be made, according to Fig. 2 and p. 363.

TABLE IV

Factors for Calculations, from Observed P_{N_2} Measurements, of Gasometric Serum Chloride Analyses, without Deproteinization

Temperature	*Macro*analysis of 0.5 cc. sample		*Micro*analysis of 0.2 cc. sample		*Micro*analysis of 0.1 cc. sample; or *ultramicro*analysis of 0.05 cc. sample		*Ultramicro*analysis of 0.02 cc. sample	
	$d = 20$		$d = 26.05$		$d = 31.05$		$d = 31.15$	
	Supernatant sample = 1.0 cc.						Supernatant sample = 0.5 cc.	
	$a = 0.5$ cc. $K = 0.985$							
	Factor by which P_{N_2} is multiplied to find							
	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.
°C.								
15	0.3780	2.211	0.4925	2.881	0.587	3.434	1.173	6.86
16	66	03	05	70	85	22	69	84
17	52	2.195	0.4890	61	83	11	64	81
18	38	87	70	49	81	3.399	60	79
19	24	79	50	37	79	87	56	76
20	0.3711	2.171	0.4835	2.828	0.577	3.375	1.152	6.74
21	0.3698	63	15	17	75	64	48	72
22	85	56	00	08	73	52	43	69
23	71	48	0.4785	2.799	71	40	39	66
24	58	40	65	88	69	29	35	64
25	0.3646	2.133	0.4750	2.779	0.567	3.317	1.131	6.62
26	34	26	35	70	65	05	27	59
27	22	19	20	61	63	3.294	24	57
28	09	11	00	50	61	82	20	55
29	0.3597	04	0.4685	41	59	70	16	53
30	0.3585	2.097	0.4670	2.732	0.557	3.258	1.112	6.51
31	73	90	55	23	55	47	08	48
32	59	82	35	11	53	35	04	46
33	47	75	20	03	51	23	1.100	44
34	35	68	05	2.694	49	12	1.096	41

The factors have been derived from Equation 5.

Table V

Factors for Calculations, from Observed P_{N_2} Measurements, of Gasometric Chloride Analyses of Serum and Whole Blood Filtrates

Temperature	Tungstic acid serum filtrate		Zinc hydroxide serum filtrate		Tungstic acid blood filtrate; *macro*analyses of 1 cc. sample or *micro*-analyses of 0.2 cc. sample	
	$d = 10$		$d = 11$*		$d = 25$	
	Supernatant sample = 2.0 cc.†				Supernatant sample = 1.0 cc.	
	$a = 2.0$				$a = 0.5$	
	$K = 1.017$		$K = 0.995$		$K = 1.000$	
	Factor by which P_{N_2} is multiplied to find					
	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.
°*C.*						
15	0.3655	2.138	0.4110	2.404	0.4656	2.724
16	42	31	0.4094	2.395	38	13
17	28	22	79	86	21	03
18	14	14	63	77	03	2.693
19	01	07	49	69	0.4587	83
20	0.3588	2.099	0.4034	2.360	0.4571	2.674
21	76	92	20	52	54	64
22	63	84	05	43	38	55
23	50	77	0.3991	35	22	45
24	37	69	77	27	05	35
25	0.3525	2.062	0.3963	2.318	0.4490	2.627
26	14	56	50	11	75	18
27	02	49	37	03	60	09
28	0.3490	42	23	2.295	45	00
29	78	35	10	87	30	2.592
30	0.3466	2.028	0.3897	2.280	0.4415	2.583
31	54	21	84	72	00	74
32	42	14	69	63	0.4384	65
33	30	07	56	56	69	56
34	18	00	43	48	54	47

The factors have been derived from Equation 5.

* If the serum filtrate + H_3PO_4 mixture is prepared by diluting 9 volumes of filtrate to 10 volumes (see foot-note 13), $d = 11.11$ and the factors are multiplied by 1.010.

† If supernatant samples of 1 cc. are used, the results are multiplied by 2.004. If supernatant samples of 0.5 cc. are used, the P_{N_2} measurements are made at $a = 0.5$ cc. volume, and the results are multiplied by 0.998.

prescribed for the ultramicroanalysis of serum, would have to be used. The amount of air in the blank would then be increased, as would the time necessary to carry out an analysis. The writer has preferred to use the mercury seal, to determine accurately the loss involved in sampling, and to apply a corresponding correction to the results.

The correction was determined as follows: A sample of 1 N NaOH was taken in the pipette to be used for iodate determinations. The tip was *thoroughly* washed in a stream of distilled water, and the sample was delivered into the chamber through the mercury seal in the cup, as described above. The mercury was run down nearly to the capillary of the cup. The layer of water above was stirred with a rod, then titrated with 0.01 N HCl, with alizarin sulfonate as indicator. The results varied slightly, depending on the combination of pipette and apparatus used. A loss of from 0.003 to 0.005 cc. was found.

In the forms of extraction chambers used for this work the cups were sealed onto the capillary in such a manner that the bottom of the cup was relatively flat and not pointed. The rubber tip on the pipettes used was also flat bottomed, so that it fitted snugly to the cup at the point where the capillary suddenly widened out. Under these conditions the minimum losses mentioned above were observed.

The results of the present paper have been corrected for these errors. In order that a separate calculation may be avoided, an average correction factor *m* of 1.004 and 1.002, for samples of 1 and 2 cc., respectively, has been incorporated in the tables of factors (Tables III, IV, and V) for use with this method. However, it is strongly recommended that the analyst determine for himself the error involved in the use of any particular pipette and extraction chamber. Should the sample loss be sensibly different from that given above, a further correction should be applied to all results calculated according to Tables III, IV, and V. Thus, if it be found that there is a 0.7 per cent loss on delivery of a 1 cc. sample, all of the results should be increased by a factor of 1.003 over the values calculated according to the appropriate table of factors.

Derivation of Factors of Tables III, IV, and V—The symbols used in the following equations are:

$P =$ mm. N_2 pressure measured in the gasometric iodate analysis of the supernatant fluid from the $AgIO_3$ precipitation of chloride

$a =$ cc. volume at which P is read in the extraction chamber

$s =$ " " of supernatant sample analyzed

$S =$ " total volume of solution extracted in the extraction chamber

$[N_2] =$ mM nitrogen per liter of supernatant sample, found by iodate analysis

$[IO_3]_e =$ mM iodate per liter of original, undiluted sample found by analysis of supernatant sample

$[Cl]_i =$ mM chloride per liter of original, undiluted sample

$f =$ factor converting values of P to values of $[N_2]$, when $s = 1.0$, $S = 3.5$, and $a = 2.0$

$F = f/3$

$d =$ dilution factor, for the number of times the original sample volume was increased by dilution, before being shaken with $AgIO_3$

$m =$ correction factor, for loss of sample in transfer through mercury seal

$K = \frac{[IO_3]_e}{[Cl]_i} =$ empirical reaction yield factor expressing ratio of the concentration of iodate found by analysis to the concentration of chloride originally present, per liter of original sample

From Table III of Van Slyke and Neill (1924) the mM of N_2 obtained from the analysis of the supernatant iodate-containing solution may be calculated. We may use as f their factor for nitrogen from a 1 cc. sample, with solution volume $S = 3.5$ cc., and with the gas measured at the 2.0 cc. volume ($a = 2.0$ cc.).[14] Then, with N_2 measured at any volume, a, mM of N_2 per liter of supernatant sample analyzed will be

$$[N_2] = \frac{P \times f \times a \times m}{2 \times s} \tag{3}$$

To calculate iodate from N_2, the factor $\frac{2}{3}$ is introduced, since 2 moles of iodate yield 3 moles of N_2 (Equation 2). Furthermore, the dilution factor d is used to calculate values in terms of concentrations in the original, undiluted sample.

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[14] A change in value of S, the total amount of liquid in the chamber when the nitrogen gas is evolved, has but slight effect on these calculations (0.1 per cent difference between $S = 2.5$ and 5.0), and hence may be neglected. However, S must always be exactly the same when p_0 is determined in the blank analysis as when p_1 is determined in the iodate analysis.

$$(4) \qquad [IO_3]_e = \frac{2}{3} \times \frac{P \times f \times a \times m \times d}{2 \times s} = \frac{f}{3} \times \frac{Pamd}{s}$$

To calculate the chloride from the iodate values, it is necessary to introduce the factor K, which is slightly less than unity, for the reason that under the usual conditions of analysis 1 mole of chloride yields slightly less than 1 of iodate. For pure chloride solutions under such conditions the value of K has values both theoretically and empirically of from 0.996 to 1.000 (Tables VI and XI). For urine, plasma, and plasma or blood filtrates, however, K has a slightly different, empirical value, which is used in the calculations for each of these materials, and which has been

TABLE VI

Theoretical Calculations of Concentrations at Equilibrium of the Reaction in Water: $AgIO_3 + NaCl \rightleftarrows NaIO_3 + \downarrow AgCl$

$[IO_3^-]_e$ = mM total iodate in solution per liter at end of reaction. $[Cl^-]_i$ = mM chloride per liter, *initial*, at beginning of reaction. $[Cl^-]_e$ = mM chloride per liter, *final*, at end of reaction. $[IO_3^-]_s$ = mM iodate per liter, as physically dissolved $AgIO_3$, at end of reaction.

$[IO_3^-]_e$	From Equation			Ratio $\frac{100[IO_3^-]_e}{[Cl^-]_i}$
	8	7	16	
(1)	$[Cl^-]_e$ (2)	$[IO_3^-]_s$ (3)	$[Cl^-]_i$ (4)	(5)
1.00	0.0043	0.0400	0.964	103.70
1.25	0.0054	0.0320	1.223	102.17
1.50	0.0065	0.0267	1.480	101.36
2.00	0.0086	0.0200	1.989	100.57
2.50	0.0108	0.0160	2.495	100.21
3.00	0.0129	0.0133	3.000	100.00
3.50	0.0151	0.0114	3.504	99.90
4.00	0.0173	0.0100	4.007	99.82
5.00	0.0216	0.0080	5.014	99.73
6.00	0.0259	0.0067	6.019	99.68
8.00	0.0345	0.0050	8.030	99.63
10.00	0.0432	0.0040	10.039	99.61
12.00	0.0518	0.0033	12.048	99.60

incorporated in the computation of the factors of Tables III, IV, and V. Therefore, the mM of chloride per liter of *original* sample will be

$$[\text{Cl}]_i = P \times \left(\frac{Famd}{K_S}\right)^* \tag{5}$$

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* If values in terms of gm. of NaCl per liter are desired, the values of mM of chloride per liter are multiplied by the factor 1/17.1 or 0.0585. In terms of mg. of NaCl per 100 cc., values of mM of chloride per liter are multiplied by 5.85.

The calculated values for F are given in Table III. From these values, with the aid of Equation 5, values for chloride content of samples analyzed under any of the conditions outlined may be calculated. Obviously, from Equation 5 it is possible to combine the various factors for any one set of conditions and thus to calculate a *single* factor (enclosed in parentheses) by which to multiply the nitrogen pressure to obtain chloride concentrations directly. Such single, composite factors are given in Tables III, IV, and V for use with urine, plasma, or protein-free filtrate analyses.

Example—A sample of 0.5 cc. of *serum* was diluted to 10 cc. before addition of silver iodate. Of the centrifuged, supernatant liquid, 1 cc. was delivered through a mercury seal, into the extraction chamber. The p_1 reading measured at 0.5 cc. volume was 371.5 mm. at 25°. The blank p_0 reading was 96.1 mm. at 24.5°. According to Table II the corrected p_0 reading was 96.8 mm. at 25°. Thus $p_1 - p_0 = P = 274.7$ mm. According to Equation 5 the chloride concentration of the original sample is calculated to be

$$\frac{274.7 \times 0.03578 \times 0.5 \times 1.004 \times 20}{0.985 \times 1.0} = 100.2 \text{ mM Cl per liter}$$

The simple calculation by the use of the composite factor for these conditions (Table IV, second column) is merely 274.7 × 0.3646 = 100.2 mM of Cl per liter.

Correction for Solubility of $AgIO_3$ in Routine Method for Urine—As is noted in the following "Theoretical" and "Experimental" sections, the effect on the results of the solubility of $AgIO_3$ becomes significant when the iodate concentration of the supernatant fluid in the chloride analysis is less than 3 mM. In the *routine* analysis of urine, when samples containing less than 60 mM of chloride per liter (or 3.51 gm. of NaCl per liter) are diluted 20-fold, a solubility correction for $AgIO_3$ must, therefore, be applied to the results after calculation according to Table III. These corrections are given in Fig. 2, where the curves indicate the corrections to be subtracted from any given total chloride value obtained by the

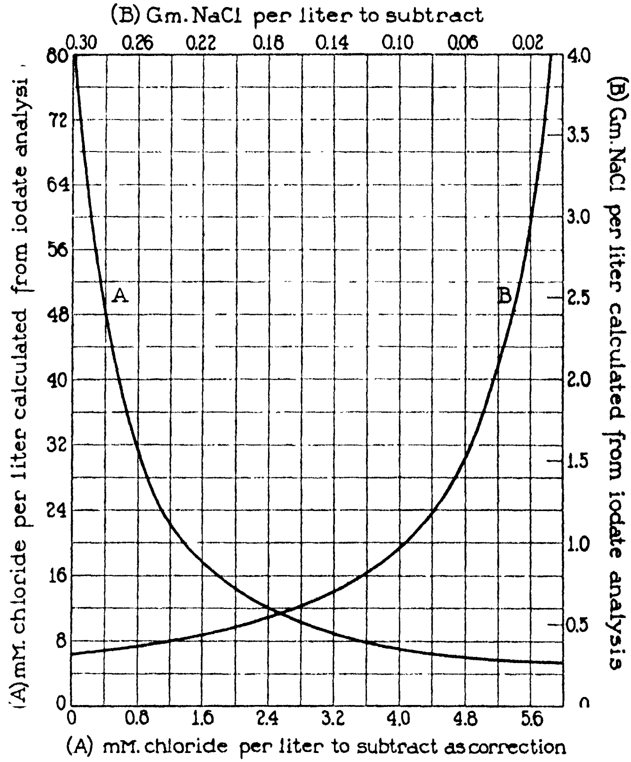

FIG. 2. Corrections to be subtracted from the results of routine urine chloride analyses when calculation according to Table III indicates less than 60 mM of chloride per liter (or 3.2 gm. of NaCl per liter) for the undiluted urine.

routine procedure. *The curves of Fig. 2 apply only to urine diluted 20-fold with 0.17 M H_3PO_4.* The derivation of the correction curves is discussed in the "Experimental" section following.

Example—A sample of nephritic urine was found by *routine analysis* and calculation from Table III to contain apparently 12.00 mM of chloride per

liter. Since the value was less than 60 mM (and therefore less than 3 mM for the diluted analyzed sample), the correction of Curve A of Fig. 2 was applied. The corrected chloride concentration of the undiluted urine was, therefore, 12.00 − 2.40 = 9.60 mM per liter.

THEORETICAL

Upon the completion of the reaction upon which this method is based (Equation 1), a solution will be in equilibrium with both solid silver chloride and solid silver iodate. According to the law of mass action, with complete dissociation of the dissolved silver salts, the relationships for the equilibria will be

$$[Ag^+] \times [Cl^-] = K_{AgCl} \tag{6}$$

$$[Ag^+] \times [IO_3^-] = K_{AgIO_3} \tag{7}$$

where the brackets indicate concentrations in mM per liter, and K_{AgCl} and K_{AgIO_3} indicate the solubility product constants.

In the present method the reaction takes place in acid solutions, usually of 0.085 M phosphoric acid. If the possible effect on solubility of the acidity and other changes in the chemical composition of biological fluids be neglected, one may approximately calculate solubility products in such a medium from the available values for solubility in water. From Brown and MacInnes (1935) the solubility of silver chloride in water at 25° is 0.01314 mM per liter. From the Landolt-Börnstein-Roth "Tabellen" (1912) the solubility of silver iodate at that temperature is 0.19 mM per liter. From our own determinations at the same temperature, this value is 0.20 mM per liter.

From the above values one obtains at 25° values of $K_{AgCl} = (0.01314)^2 = 0.00017266$, and $K_{AgIO_3} = (0.200)^2 = 0.04$. Substituting in Equations 6 and 7, and combining, one obtains

$$\frac{[Cl^-]_e}{[IO_3^-]_e} = \frac{K_{AgCl}}{K_{AgIO_3}} = 0.004316 \tag{8}$$

as the expression for the relative final concentrations of chloride and iodate ion in water in equilibrium with both solid silver salts.

In the reaction of Equation 1 the difference between the initial chloride concentration, $[Cl^-]_i$, and the final, $[Cl^-]_e$, is equal to the concentration of iodate, $[IO_3^-]_r$, dissolved from silver iodate by reaction with chloride. Thus

$$(9) \qquad [Cl^-]_i = [Cl^-]_e + [IO_3^-]_r$$

Also, the iodate dissolved as the result of reaction of $AgIO_3$ with Cl is joined in the solution by a slight amount of iodate which dissolves physically as $AgIO_3$, and is indicated as $[IO_3^-]_s$. Thus

$$(10) \qquad [IO_3^-]_e = [IO_3^-]_r + [IO_3^-]_s$$

From Equations 9 and 8

$$(11) \qquad \frac{[Cl^-]_i}{[IO_3^-]_e} = \frac{[IO_3^-]_r + [Cl^-]_e}{[IO_3^-]_e} = \frac{[IO_3^-]_r}{[IO_3^-]_e} + 0.004316$$

From Equation 10 one obtains, for the relationship between the initial chloride and the final total iodate concentrations,

$$(12) \qquad \frac{[Cl^-]_i}{[IO_3^-]_e} = \frac{[IO_3^-]_r}{[IO_3^-]_r + [IO_3^-]_s} + 0.004316$$

As the concentration of iodate, $[IO_3^-]_r$, from chloride increases, the amount of iodate from physically dissolved silver iodate, $[IO_3^-]_s$, decreases, until a point is reached where $[IO_3^-]_s$ becomes vanishingly small. Equation 12 then becomes

$$(13) \qquad [Cl^-]_i = 1.004316[IO_3^-]_e$$

At this point then,

$$(14) \qquad [IO_3^-]_e = [IO_3^-]_r = [Cl^-]_i - [Cl^-]_e = 0.9957[Cl^-]_i$$

which means that the *limiting* value for the extent of the reaction of Equation 1, or the yield of iodate from reaction with chloride, will be reached when 99.57 per cent of the chloride initially in solution has been precipitated, with a corresponding liberation of iodate, mole for mole.

However, this condition is *apparently* exceeded, since in all cases there is *some* solubility of silver iodate, depending on the amount of iodate released by the chloride. Since, under these conditions, from Equations 9 and 10,

$$[Cl^-]_i = [Cl^-]_e + [IO_3^-]_e - [IO_3^-]_s \tag{15}$$

and since $[IO_3^-]_s$ can only be derived from silver iodate with solution of the *same* number of silver $[Ag^+]_s$ ions per liter, and since $[Ag^+]_s$ is defined by Equation 7, we may calculate values for $[Cl^-]_i$ entirely in terms of $[IO_3^-]_e$ to obtain another expression for Equation 11 or 12 which can be used for calculating the relationships between the initial chloride and the final total iodate concentration. That expression is

$$[Cl^-]_i = 1.004316[IO_3^-]_e - \frac{0.04}{[IO_3^-]_e} \tag{16}$$

From Equation 15 values for $[Cl^-]_i$, corresponding to analyzed values of $[IO_3^-]_e$, have been calculated for Table VI. The effect of the added iodate in the form of $[IO_3^-]_s$ is shown in Table VI in the values calculated for the ratio we refer to as the reaction yield factor K, or $[IO_3^-]_e/[Cl^-]_i$.

The *apparent* yield of iodate, $[IO_3^-]_e$, from chloride solutions of between 12 mM and 3 mM per liter would be from 99.61 to 100.00 per cent, a variation accounted for by the increase in physically dissolved silver iodate of from 0.04 to 0.44 per cent of the total iodate in the solution at equilibrium. At concentrations below 3 mM per liter of chloride the correction for dissolved $[IO_3^-]_s$ becomes significant. At 1 mM per liter of chloride in aqueous solutions it amounts to 4 per cent of the total $[IO_3^-]_e$ present at the end of the reaction.[15]

The foregoing theoretical calculations are based on the solubilities of silver iodate and chloride *in water* at equilibrium. In another solvent, such as 0.085 M or 0.17 M phosphoric acid dilutions of salt or other solutions, the solubilities of AgCl and $AgIO_3$, and consequently the solubility product ratios, may be expected to be somewhat different. Furthermore, temperature variation may also be a factor. The available data indicate that the relative solubilities of AgCl and $AgIO_3$ are little changed with variation in temperature. We have no reliable, accurate data on the effect of 0.085 M and 0.17 M H_3PO_4 on these *relative solubilities*. Our own data indicate that the solubility of $AgIO_3$, itself, is approximately 30 per cent greater in 0.17 M H_3PO_4 than in water.

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[15] For the exceptional cases in which solutions with chloride of less than 3 mM concentration may be encountered, see the "Experimental" section and the section on "Calculations," with especial reference to the *routine method* for urine.

However, it does not appear that the differences between the calculated and the real values for relative solubilities, under the usual analytical conditions of the present method, are significant. A change of 25 per cent in the value of the ratios of Equation 8 would result in a calculated yield of iodate from chloride, for chloride solutions between 12 and 3 mM, only 0.1 to 0.3 per cent different from that given by Equation 16. The experimental results bearing on the extent of the reactions involved, and on the question of correcting for dissolved iodate, will be considered in the following sections.

EXPERIMENTAL

Solubility of Silver Iodate—The determination of the solubility in water is suggested ("Reagents") as one of the two tests of the purity of the silver iodate. The experiments done in this connection are detailed in the following. Saturated silver iodate solutions were prepared by shaking 15 cc. portions of water with $AgIO_3$ for 30 minutes at 25°. The solutions were filtered or centrifuged. The dissolved iodate was determined both gasometrically and by titration.

For the *gasometric* determination, 5 cc. of hydrazine and 5 cc. of filtrate were measured into the extraction chamber. The chamber was evacuated and the solution was shaken for 3 minutes. The gas pressure was then read at the 0.5 cc. volume for p_1. The p_0 reading was obtained in a blank analysis in which water replaced the saturated solution of $AgIO_3$. A pressure of about 51 mm. of N_2 from dissolved $AgIO_3$ was obtained.

Titrimetrically (Sendroy, 1937, *a*) a more accurate result was obtained by titrating 3 cc. samples of filtrate with 0.0012 N $Na_2S_2O_3$, of which about 3 cc. were required. Before the titration was begun, 5 drops of 0.85 M H_3PO_4 and 1 drop of 5 per cent KI were added. Near the end-point 1 drop of 0.5 per cent starch solution was added. The thiosulfate solution was standardized with 0.0012 N KIO_3 (= 0.0002 M) in place of the sample.

The results of Table VII indicate that under these conditions, the solubility of silver iodate in water is 0.20 mM per liter, a value that is constant for saturation obtained by shaking between 30 and 90 minutes. Upon the addition of phosphoric acid, the $AgIO_3$ solubility increased to 0.25 to 0.26 mM per liter in 0.17 M H_3PO_4.[16]

As has been noted in the preceding "Theoretical" section, the solubility of $AgIO_3$ in the supernatant fluid of the chloride analysis

TABLE VII

Results of Solubility Determinations in Water and in H_3PO_4 Solutions at 24-25° of Several Lots of $AgIO_3$

Lot No.	Solvent	Time of shaking	Method of iodate determination	Solubility of $AgIO_3$
		min.		*mM per l.*
1	H_2O	30	Gasometric	0.20
		30	"	0 20
		60	"	0 20
		60	"	0.19
		60	"	0 19
2	"	30	"	0.19
		60	"	0.21
		60	"	0.20
		60	"	0 21
		90	"	0 20
3	"	3	Titrimetric	0.19
		30	Gasometric	0 19
		40	"	0 20
3	0 085 M H_3PO_4	3	Titrimetric	0 24
		3	Gasometric	0.23
		30	"	0.23
		40	"	0 23
		40	Titrimetric	0 24
		60	Gasometric	0 24
3	0 128 " "	3	"	0 24
3	0 17 " "	3	"	0 25
		3	"	0 26

is in all cases much less than these values, because the $NaIO_3$ formed depresses the $AgIO_3$ solubility. Within the concentration limits of 12 and 3 mM chloride, the $AgIO_3$ solubility is negligible, as is indicated by the calculated values for dissolved $AgIO_3$ in aqueous solutions of NaCl (Table VI, Column 3). We have in fact been unable to measure any definite increase in the iodate concentrations of known solutions of KIO_3 in 0.085 M H_3PO_4 of the concentrations formed in serum chloride analysis, when they were shaken with $AgIO_3$ for 3 minutes (Table VIII). Under these conditions, therefore, no correction for $AgIO_3$ solubility is required.

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[16] These results were later confirmed by the colorimetric technique (Sendroy, 1937, *b*), the accuracy of which is equal to that of the titrimetric method for the determination of iodate in such dilute concentrations.

TABLE VIII

Effect of Shaking Iodate Solutions in 0.085 M Phosphoric Acid, with Solid $AgIO_3$ for 3 Minutes at 24–25°. Gasometric Measurements

$AgIO_3$	Iodate		$AgIO_3$ solubility	
	Present in $KH(IO_3)_2$ solution	Found by analysis	Found by difference of averages	Theoretical for unacidified aqueous solution according to Table VI
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*
None	10.00	10.00		
		10.00		
		10.04		
Added		10.00	−0.01	+0.004
		10.02	+0.01	+0.004
None	2.50	2.500		
		2.503		
		2.497	−0.004	+0.016
Added		2.509	+0.008	+0.016
		2.504	+0.003	+0.016

On the other hand, in more dilute iodate solutions, such as may be obtained in the *routine method* applied to low chloride urines, the solubility of $AgIO_3$ becomes theoretically greater. The effect of this increased solubility on the yield of total dissolved iodate from small concentrations of chloride can be demonstrated experimentally. The results are discussed in connection with the analysis of low chloride nephritic urines.

Determination of Chloride in Known Salt Solutions—In the study of the various factors affecting the reaction of chlorides with silver iodate, several hundred analyses were carried out on solutions of known chloride content. The starting material was always a 0.1 M chloride solution which was diluted from 10 to 30 times for the determinations. Chlorides of reagent grade, obtained from various sources, were used. Some were used directly from the container as purchased, others were dried or recrystallized several times, and one was fused after three recrystallizations. The results with all were found to be the same within the limits of error. Since the large number of analyses made makes it impractical to list all of the results, the tables illustrating the various significant points should be considered as merely representative of a still larger number of results not reported.

Effect of pH—Chlorides are usually precipitated in an acid medium in order to prevent the precipitation of other insoluble silver salts, and to obtain a good coagulation of the precipitated material. In unacidified salt solutions diluted to 10 mM of chloride per liter, the silver chloride, at least, remained partially in colloidal form and caused slightly high results. On the other hand, nitric acid, in the presence of which chlorides are usually precipitated, could not be used for work in the Van Slyke apparatus, since it reacts with mercury.

Table IX indicates the results of a large number of determinations made in a variety of diluents, as a test of their suitability for work with biological materials. In duplicate analyses two separate portions of standard chloride solution were shaken with $AgIO_3$. The supernatant liquid in all cases, except when water alone was the diluent, was almost clear. In inorganic chloride solutions of pH less than 6.5 phosphates were not found to interfere with the reaction between chloride and silver iodate. Of other substances added, only bicarbonate in solutions above pH 6.2 was found to interfere; it caused solution of 1.008 moles of iodate per mole of chloride. All of the other results were close to the theoretically calculated yield of 99.6 per cent (Equation 15) for 0.01 M unacidified aqueous chloride solutions.

Amount of Silver Iodate Required—By calculation, 10 cc. of 0.01 M chloride solution require 0.0283 gm. of $AgIO_3$. In order to provide a sufficient excess, experiments were carried out to determine the iodate yield from 0.01 M solutions of NaCl in 0.085 M H_3PO_4, to which different amounts of $AgIO_3$ were added. The results are as follows:

$AgIO_3$ per 10 cc. solution, *gm.*	0.03	0.06	0.12
IO_3 per liter found, *mM*	9.48	10.00	9.98

From this it is apparent that twice the calculated necessary amount of $AgIO_3$ was adequate for complete reaction (theoretical yield in acidified solution = 99.6 per cent). Our procedure calls for the use of what is equivalent to at least 3 times the stoichiometrical amount. Use of more has no further effect other than to make the supernatant liquid slightly clearer in some solutions.

TABLE IX

Effect of pH on Silver Iodate Determination of Chloride in 0.01 M Solutions of Sodium Chloride at Room Temperature. Gasometric Results

No. of solutions analyzed	Diluent in which NaCl was present as 0.01 M	pH	Average chloride concentration found by analysis
			mM per l.
4	Water	6.6	10.01
5	0.1% acetic acid	3.7	9.99
3	0.5% " "	3.2	9.96
2	M/15 acetate mixture	4.0	9.95
2	M/15 " "	4.5	9.97
2	M/15 " "	5.0	10.01
2	M/15 " "	5.5	10.00
89	0.085 M H_3PO_4	2.1	9.98
3	0.17 " "	2.0	9.98
20	Tungstic acid mixture	2.4	9.99
46	M/15 KH_2PO_4	4.5	9.98
4	M/15 phosphate mixture	5.5	9.98
4	M/15 " "	6.5	10.01
4	M/15 " "	7.5	10.00
4	M/15 " "	8.5	10.01
6	M/15 phosphate + M/15 $NaHCO_3$	6.2	10.07
4	M/15 " + M/15 "	6.8	10.09

Time Necessary for Completion of Reaction of $AgIO_3$ with Chloride—Solutions of 0.1 M NaCl diluted to 10 to 40 times with 0.085 M H_3PO_4 were shaken with $AgIO_3$ (0.1 gm. per 10 cc. of solution) for varying lengths of time, from ½ minute to 1 hour. Table X shows that the reaction was nearly complete in ½ minute.

The recommended procedure, of a minimum 2 minutes shaking with $AgIO_3$, allows a wide margin of safety for the completion of the reaction when the chloride concentration is between 2.5 and 10 mM.

Effect of Temperature on Reaction of $AgIO_3$ with Chloride—Solutions of 0.1 M NaCl were diluted to 10 times with 0.085 M phosphoric acid, then brought to thermal equilibrium by shaking in tubes kept at different constant temperatures. Then $AgIO_3$ was introduced, and the shaking was continued at the same temperatures, for times ranging from ½ to 10 minutes. Repeated experiments carried out at temperatures ranging from 15–35° failed to show any consistent effect of temperature on the reactions.

TABLE X

Effect of Time of Shaking with $AgIO_3$ on the Reaction: $NaCl + AgIO_3 \rightleftarrows \downarrow AgCl + NaIO_3$ As Found by Gasometric Measurements

Experiment No.	Chloride concentration	Time of shaking with $AgIO_3$							Theoretical ratio $\frac{100[IO_3]_e}{[Cl]_i}$ for unacidified aqueous solutions
		½ min.	1½ min.	3 min.	5 min.	10 min.	30 min.	60 min.	
		Extent of reaction as indicated by ratio, $100 \times \frac{\text{mM per liter iodate found}}{\text{mM per liter chloride present}}$							
	mM per l.								
1	10.00				99.9	100.0			99.6
2	10.00				99.3	99.9	99.8	99.6	
3	10.00		99.7	99.3					
4	10.00				99.9	99.9	99.7	99.9	
5	10.00	99.5	99.5						
6	10.00	99.4	99.6						
7	5.00		99.5		99.4	99.8	100.2	99.6	99.7
8	5.00	99.5	100.0						
9	5.00	99.5	99.6						
10	2.50		100.5		100.3	100.2	100.3	99.8	100.2
11	2.50			99.9	99.7				
12	2.50		99.7	100.4			99.5	99.8	

The results of a typical experiment with a 10 mM chloride solution were as follows:

Temperature, °*C*......	15	20	25	33	35
Iodate per liter found, *mM*..........	9.	9.99	9.99	9.94	9.96

No solubility data are available for the calculation of the equilibrium (Equation 8) at various temperatures other than at 20° and 25°. Theoretically, the difference between the iodate yield, relative to the initial chloride concentration, at 20° and at 25° is about 0.1 per cent. Within ordinary limits of room temperature, therefore, the effect of variations in the temperature may be considered negligible.

TABLE XI

Summary of Results of Chloride Determinations in 0.085 M Phosphoric Acid Solutions of Known Composition, at Varying Dilutions

Chloride present = $[Cl]_i$ (1)	Method of iodate analysis (2)	No. of solutions analyzed (3)	Measured yield of total iodate from chloride, ratio $\frac{100[IO_3^-]_e}{[Cl^-]_i}$: Range (4)	Mean value of all results (5)	Average deviation from mean (6)	Theoretical value for unacidified aqueous solution (Table VI) (7)
mM per l.						
10.00	Gasometric	89	99.1–100.5	99.8	±0.26	99.6
	Titrimetric	5	99.2– 99.9	99.5	±0.24	
	(Colorimetric)		(99.2– 99.6)	(99.4)		
8.00	Gasometric	11	99.2– 99.9	99.6	±0.23	99.6
7.50	"	4	99.6–100.0	99.8	±0.18	99.6
6.00	"	6	99.2–100.4	99.9	±0.30	99.7
5.00	"	24	99.3–100.4	99.8	±0.24	99.7
	Titrimetric	8	99.3–100.0	99.6	±0.20	
	(Colorimetric)		(99.2–100.0)	(99.6)		
4.00	Gasometric	37	99.3–100.5	100.0	±0.24	99.8
	Titrimetric	29	99.1–100.4	99.8	±0.24	
	(Colorimetric)			(100.5)		
3.00	Gasometric	8	99.3–100.3	100.0	±0.24	100.0
	Titrimetric	15	99.4–100.4	100.0	±0.23	
2.50	Gasometric	8	100.0–101.1	100.2	±0.44	100.2
	(Colorimetric)		(99.6–100.0)	(99.8)		
2.00	Gasometric	10	100.0–101.0	100.5	±0.26	100.6

Effect of Variation in Chloride Concentration on the Reaction: $AgIO_3 + NaCl \rightleftarrows \downarrow AgCl + NaIO_3$—From theoretical considerations, with decrease in chloride concentration one may expect an increased physical solubility of silver iodate. With sufficient dilution of the chloride, the physically dissolved $AgIO_3$ added to the formed $NaIO_3$ is sufficient to increase measurably the amount of total iodate dissolved per mole of chloride (Table VI). The actual $AgIO_3$ solubility itself, since it is so small (Table VI, Column 3) under these conditions, is a difficult thing to demonstrate (Table VIII) in any simple analysis of chloride solutions between 2.5 and 10 mM, but its *increase* with decrease in chloride concentration becomes apparent in the average results of a large number of analyses, as is shown by the results of Table XI. In the previous "Theoretical" section it was also pointed out that the increase in solubility of $AgIO_3$ in acid medium over that in neutral water would result in a slight increase of values for the moles of total iodate obtained per mole of chloride. In fact, the results of Table XI (Column 5) average slightly more than 0.1 per cent higher than the corresponding calculated values for the same concentrations of chloride in unacidified water solutions (Column 7).

TABLE XII

Distribution of Some Results of Table XI for Gasometric and Titrimetric Analyses of Known Chloride Solutions in 0.085 M H_3PO_4

Chloride concentration	No. of solutions analyzed	Mean concentration of dissolved iodate found	Percentage distribution of results for total dissolved iodate found for limits of variation from mean, of					
			±0.0%	±0.1%	±0.2%	±0.3%	±0.5%	±0.7%
mM per l.		*mM per l.*						
10.00	94	9.98	11	36	54	71	95	100
4.00	66	3.996	15	47	64	74	93	100

The results of Table XI, which include only determinations of chloride in phosphoric acid solutions, are indicative of the reliability of the method for inorganic salt solutions. The average deviation from the mean for the measured values of Column 5 is about ±0.24 per cent for both the *gasometric* and *titrimetric* (Sendroy, 1937, *a*) determinations. The *colorimetric* (Sendroy, 1937, *b*) results have shown a mean variation of about ±1.0 per cent of the theoretical, with limits of ±2.0 per cent.

The distribution of results of the two larger groups of analyses is shown in Table XII.

Effect of Various Substances on Reaction of $AgIO_3$ with Chloride

TABLE XIII

Effect of Various Substances on Reaction of $AgIO_3$ with Chloride or Other Halides

Group No.	No. of solutions analyzed	Composition of diluted halide solution	pH of diluted halide solution	Method of iodate analysis[*]	Total dissolved iodate from halide, ratio $\frac{100[IO_3]_e}{[\text{halide}]_i}$	
					Found by analysis	Theoretical in unacidified halide solution
1	2	10 mM KBr, 0.085 M H_3PO_4	2.1	G.	100.2	100.0
	2	4.895 mM KBr, 0.168 mM KCl, 0.085 M H_3PO_4	2.1	"	99.9	100.0
2	4	10 mM KI, 0.061 M KH_2PO_4	4.5	"	100.0	100.0
	2	1 " " 9 mM NaCl, 0.085 M H_3PO_4	2.1	"	95.7	99.6
	2	1 " " 9 " " 0.085 " "	2.1	T.	95.7	99.6
	2	0.50 mM KI, 9.50 mM NaCl, 0.085 M H_3PO_4	2.1	G.	98.5	99.6
	2	0.25 " " 9.75 " " 0.085 " "	2.1	"	99.2	99.6
3	4	10 mM NH_4Cl, 0.061 M KH_2PO_4	4.5	"	99.0	99.6
4	2	10 " HCl, 0.2 M acetate mixture	3.9	"	99.6	99.6
	6	10 " " 0.01 " KH_2PO_4, 0.085 M H_3PO_4		"	99.6	99.6
	2	10 " " 0.085 " H_3PO_4		T.	99.5	99.6
	2	5 " " 0.085 " "		"	99.6	99.7
5	2	10 " NaCl, 2 mM $CaCO_3$, 0.17 M H_3PO_4	2.0	G.	99.9	99.6
6	2	10 " " 2 " $Na_2C_2O_4$, 0.17 M H_3PO_4		"	99.6	99.6
	2	5 " " 0.05% $K_2C_2O_4$, 0.085 M H_3PO_4		"	99.9	99.7
	2	5 " " 0.05% " 0.085 " "		T.	100.0	99.7
7	2	5 " " 0.05% Na citrate, 0.085 M H_3PO_4		G.	100.0	99.7
	2	5 mM NaCl, 0.05% Na citrate, 0.085 M H_3PO_4		T.	100.0	99.7
8	2	5 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		G.	100.2	99.7
	2	4 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		T.	99.8	99.8
	2	4 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		G.	100.0	99.8
9	4	5 mM arginine hydrochloride, 0.085 M H_3PO_4		"	99.6	99.7
	4	5 mM NaCl, 5 mM alanine, 0.085 M H_3PO_4		"	100.2	99.7
	4	5 " " 5 " glycine, 0.085 " "		"	100.2	99.7
10	4	10 " " 0.1% glucose, 0.085 M H_3PO_4		"	99.8	99.6

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[*] G. = gasometric; T. = titrimetric.

or Other Halides—The data of Table XIII supplement those of Table IX. The following conclusions are apparent. Bromides may be analyzed quantitatively by the same technique as for chloride (Group 1). Since silver bromide is much more insoluble than chloride, the reaction of bromide with $AgIO_3$ may be expected, according to solubility product relationships, to go even more nearly to completion, so that the yield of iodate per mole of bromide is 1.000 mole instead of the 0.996 mole per mole of chloride. As calculated from AgI solubility in water, the same is true for iodide. However, the experimental results show that iodides (Group 2) in this reaction are extremely sensitive to strong acid, with the result that the iodate found in solution is low, if the pH is much below 4.5. As has been shown, chlorides in inorganic salt solutions (and presumably bromides, also) in their reaction with $AgIO_3$ are unaffected by pH changes over a wide range (Table IX). When iodide is present in a mixture of halides, it is desirable to keep the pH at 4.5. On the other hand, this is too high a pH for the analysis of chloride in biological material by this method. In such solutions the presence of iodide at pH 4.5 may give rise to slightly high total halide results. Iodide is rarely present, however, in urine or plasma in amounts sufficient to necessitate any departure from the prescribed technique of diluting with phosphoric acid.

Other results of Table XIII indicate that the ammonium ion (Group 3) is without effect, and that hydrochloric acid may be standardized simply by diluting with 0.085 M H_3PO_4 and analyzing titrimetrically or gasometrically (Group 4).

The presence of calcium, oxalate, citrate, uric acid, glucose, and some amino acids, in concentrations exceeding several times the amounts usually present in urine or plasma when diluted with phosphoric acid according to the prescribed procedure, showed no significant effect on the reaction of chloride with $AgIO_3$. The variation in results was within the limits found for the results of Table IX.

Procedures Used in Testing Applicability of the Silver Iodate Method to Blood and Urine

The method has been tested in two ways: (1) Its results have been compared with those of chloride methods that have been accepted as standard procedures in general use. (2) In the case of plasma, the material has been freed of halogens by dialysis, and known amounts of NaCl have then been added. Chlorides were determined by the silver iodate method. Comparison of the results with the known amounts of NaCl added, afforded, for the accuracy of the iodate method, a test which was independent of the exactness of any other analysis. This rigid test was applied by Van Slyke (1923–24) to the nitric acid digestion method for plasma chloride, but so far as the writer is aware, with the exception of the colorimetric procedure of Westfall, Findley, and Richards (1934), it has not been applied to any of the other numerous blood chloride methods that have appeared in the literature.

Comparison with Chloride Determined by Standard Methods—Two standard chloride methods have been used, those of Van Slyke (1923–24) and of Sunderman and Williams (1933). In the Van Slyke method a standard solution of $AgNO_3$ in concentrated HNO_3 is added to the chloride-containing fluid, the organic matter is destroyed by digestion at 100° or by boiling, and the excess silver is titrated with sulfocyanate by Volhard's procedure, under the conditions adopted by Whitehorn (1920–21). In Sunderman and Williams' modification the same nitric acid digestion in the presence of $AgNO_3$ is used; but it is preceded by digestion in alkali to prevent the possibility that any chloride may be occluded by fat particles resistant to HNO_3. Furthermore, the $AgNO_3$ is added in aqueous solution before the HNO_3, as recommended by Wilson and Ball (1928). The Sunderman and Williams method, therefore, involves the successive addition of three reagents in place of the single $AgNO_3$-HNO_3 solution used by Van Slyke.

The author, in agreement with Eisenman (1929), has never encountered any significant error in analysis of plasma or serum by simple Van Slyke technique (see discussion in Peters and Van Slyke (1932) p. 831). The results were usually the same as by the Sunderman-Williams procedure, within the limits of titration error. In some sera the Sunderman-Williams method has given slightly higher results, but never by more than 1 part in 200. For comparison with the silver iodate method of serum analysis, both of the digestion methods have been used.

For some samples of *whole blood* the precautionary additions made by Sunderman and Williams to the Van Slyke procedure raise by as much as 1 per cent the chloride values found; the range of the effect has been from 0.0 to 1.0 per cent. There is no exact proof that the higher values are the more accurate, but it appears probable that they are. For comparison with the iodate method in whole blood we have, therefore, used the Sunderman-Williams technique exclusively.

In *urine*, where there are no significant amounts of lipid material or solid tissues to demand resolution by alkaline digestion, we have used the Van Slyke (1923–24) method for comparison. Harvey's (1910) application of the simple Volhard titration, without preliminary nitric acid digestion, has been found grossly inaccurate in albuminous urines (Sendroy, 1937, *c*).

Recovery of Known Amounts of Chloride Added to Serum Previously Freed of Chloride—This procedure was followed essentially as described by Van Slyke (1923–24) for whole blood, with modifications to adapt it to the iodate method as detailed later in connection with the experiments.

Urine

Normal urines containing 92 to 268 mM of chloride per liter (= 5.4 to 15.7 gm. of NaCl per liter) were diluted, as in the *precise procedure,* 10 to 25 times with 0.17 M H_3PO_4, and shaken with $AgIO_3$. The average yield of total dissolved iodate was 0.998 mole per mole of chloride present, indicated by the values for the ratio $100[IO_3]:[Cl]$ (= K) in Table XIV. In this ratio $[IO_3]$ represents the moles of iodate per liter of urine found in the sample after the reaction with $AgIO_3$, while [Cl] represents moles of chloride per liter of urine, as found by the Van Slyke (1923–24) digestion method.[17] The iodate yields for these 60 urine samples were, therefore, in complete agreement with the value of 0.9 mole of iodate per mole of chloride obtained for the known chloride solutions of Table XI.

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[17] This is consistent with the use of the ratio $[IO]_e:[Cl]_t$, or K, used in the calculations and in the experimental work with known concentrations of chloride. There [Cl], instead of being a value found by a standard method, is the true concentration fixed by the use of known amounts of pure halide salts. Thus, in general, the ratio $100[IO_3]:[Cl]$ represents that percentage of a known or analytically determined amount of halide which is measured as total dissolved iodate by the present method. The average values of K found by the precise method, 0.998 for normal urines, and 0.990 for nephritic urines, have been incorporated in the calculation of the factors of Table III.

When normal urines were analyzed by the *routine procedure*, the average of the results was the same as above. However, the probable accuracy of any individual determination was decreased by the error involved in diluting to the 10 cc. volume mark of the graduated 15 cc. centrifuge tube. These determinations were therefore in error by ±1.7 per cent.

TABLE XIV

Summary of Results of Comparative Analyses of Chloride in Human Urine by Gasometric and Titrimetric (Sendroy, 1937, a) Procedures, and by the Van Slyke (1923–24) Digestion Method

Samples	No. of samples analyzed	Ratio $\frac{100\,[IO_3]^*}{[Cl]}$			Procedure
		Average	Mean deviation from average	Extreme range	
Normal urines	37	99.8	±0.32	98.8–100.5	Gasometric, precise
	13	99.8	±0.38	98.6–100.2	Titrimetric, "
	10	99.8	±1.2	98.6–101.0	Gasometric, routine
Nephritic urines (containing protein)	36	98.9	±0.52	97.9–100.0	" precise
	25	99.1	±0.49	98.0–100.0	Titrimetric, "
	14	99.1	±3.7	92.0–107.7	Gasometric, routine
	7	99.4	±1.9	96.1–102.3	Titrimetric, "

[*] Ratio, $\frac{[IO_3]}{[Cl]}$

$$\frac{\text{mM iodate per liter urine, found by silver iodate method}}{\text{mM chloride per liter urine, found by Van Slyke digestion method}}\ K.$$

Nephritic urines (Table XIV), because of salt-restricted diets, contained 8 to 62 mM of chloride per liter (= 0.47 to 3.6 gm. of NaCl per liter). Of these urines, a few which did not contain proteins gave results by the precise procedure like those of normal urines, when compared with the Van Slyke digestion method. The others, of which 61 samples were studied, contained from 2 to 50 gm. of protein per liter. These urines yielded, on the average, 0.989 (gasometric) and 0.991 (titrimetric) mole of iodate per mole of chloride found by the digestion method. The mean deviation from these averages was about the same as for normal urines.

When these low chloride urines were analyzed by the *routine procedure,* the average error of the gasometric or titrimetric determination was about ±3 per cent, with a sample now and then showing a discrepancy of as much as ±7 per cent from the digestion method values. However, *for such low chloride material,* even this extreme error is not significant for most clinical purposes. Furthermore, although for these samples small amounts of silver nitrate and dilute NH_4CNS were used to increase the accuracy of the digestion method, the discrepancies found cannot be attributed entirely to the iodate procedure.

Sources of Error in the Silver Iodate Determination of Chloride in Urine

1. Reducing Effect of Urine on Dissolved Iodate—In the analyses of the urines summarized in Table XIV it was noticed that some very few samples, in both the normal and pathological classifications, gave slight decreasing values on each repeated analysis of the supernatant fluid. Upon further study of thirteen normal and twelve pathological urine specimens analyzed by the routine procedure, it was found that, as time elapsed after the shaking with $AgIO_3$, the iodate content of the supernatant fluid, as it stood over the centrifuged $AgIO_3$ and AgCl, decreased progressively, as shown in Fig. 3. In this respect, there seemed to be no difference between normal or pathological urines, nor could the variation in loss of iodate for different urine samples be ascribed to any known factor. *These experiments show that, if the supernatant fluid in urine analysis is analyzed within 1 hour after centrifugation, no serious error will occur from loss of iodate.*

Evidently, human urine contains material which may gradually destroy iodate under the conditions of analysis. This was further shown when KIO_3 or $KH(IO_3)_2$ was added to the dilute, acidified urine to make the iodate concentration 10 mM. Upon immediate analysis of either pathological or normal urines, 88 to 98 per cent of the added iodate was found. Two pathological urines to which iodate had been added, when analyzed gasometrically after 18 hours, yielded no nitrogen at all upon addition of the hydrazine. The disappearance of iodate in any given sample was more rapid when soluble iodate salt alone was added than when the iodate was obtained in solution by reaction of chloride with solid $AgIO_3$.

When urine is shaken with $AgIO_3$, the dissolved iodate that may be destroyed during the shaking appears to be almost completely replaced by solution of more iodate.[18] By the time the shaking period of 2 minutes is over, the iodate destruction, except in very few cases, has become sufficiently slow, so that it does not diminish the dissolved total iodate concentration significantly during the next hour (Table XIV). Hence, if the iodate determination is carried out within this time after the shaking with $AgIO_3$ is finished, significant error is avoided.

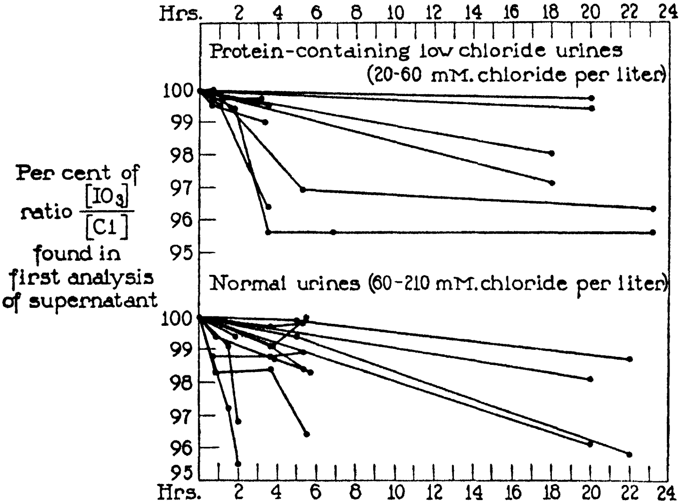

FIG. 3. Disappearance, with time, of dissolved iodate in the chloride determination of normal, and protein-containing, low chloride, nephritic urines.

2. Effect of Physical Solubility of $AgIO_3$ in the Routine Analysis of Low Chloride Urines—In addition to the possible error caused by loss of iodate in the supernatant liquid, another discrepancy which may occur in the analysis of low chloride solutions of any material makes the *routine analysis* of low chloride urines less exact than the *precise analysis.* When the chloride content of a solution is low, the physically dissolved $AgIO_3$ contributes a considerable part to the total iodate found. Furthermore, under these conditions, with 2 minutes allowed for the reaction, equilibrium of the supernatant urine with $AgIO_3$ and AgCl is only approximate. In addition, there may be a considerable variation in the solubility of $AgIO_3$ in different urines, even when diluted 20 times.

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[18] The probable reactions involved are discussed in a later section, p. 390.

It seemed best to determine empirically the apparent solubility correction for $AgIO_3$ in urine samples of varying, low chloride concentration. This was done by comparison of the results by the iodate method of a series of twenty-one different samples of low chloride urine with results by the Van Slyke digestion method. In each case the excess in the value of the iodate result over that by the digestion method was arbitrarily attributed to the solubility effect of $AgIO_3$ in the 20-fold diluted urine (Column 4, Table XV). Fairly wide discrepancies in these values, at the same chloride concentrations, were encountered. However, as might be expected, the average solubility curve (Fig. 2, "Calculations") found best suited for the entire group of the results was very nearly like that calculated for the solubility of $AgIO_3$ in solutions of chloride of these concentrations, dissolved in 0.17 M H_3PO_4. When the corrections of Fig. 2 were applied to this group of urines, results averaged within ±3 per cent of agreement with the digestion values.

That the marked variation of the solubility of $AgIO_3$ in some individual samples may account for larger differences in results is shown by an experiment carried out with Sample 19 of Table XV. This sample contained 41 gm. of protein per liter and gave distinctly positive tests for bile. (1) According to Fig. 2, the physical solubility of $AgIO_3$ in this urine diluted 20-fold, and then containing 0.75 mM of chloride per liter, would be 0.083 mM per liter. (2) From Column 4, Table XV, the apparent solubility found was 0.113 mM per liter of diluted urine or supernatant liquid. (3) When $AgNO_3$ was added to the dilute, acidified urine in concentration of 0.75 mM, just enough to precipitate the chloride, the solubility of $AgIO_3$ found was 0.33 mM per liter. (4) When to (3) there was added KIO_3 in a concentration of 0.75 mM, the solubility of $AgIO_3$ decreased to 0.13 mM per liter. (5) By calculation from the solubility product found from (3) (Equation 7) the solubility of $AgIO_3$ in a solution containing 0.75 mM of KIO_3 to begin with should be 0.125 mM per liter. The results of (2) and of (3) are in good agreement with this calculation.

TABLE XV

Effect of Physical Solubility of $AgIO_3$ on Analysis of Low Chloride Urines by the Routine Iodate Method

Sample No.	Chloride concentration in original, undiluted urine, found by analysis		$AgIO_3$ solubility effect		$AgIO_3$ method values		
	By Van Slyke method	By $AgIO_3$ routine method	Found from (3) − (2)	From Curve A, Fig. 2	Corrected according to Fig. 2, (3) − (5)	Difference from Van Slyke values	
						(6) − (2)	$(7) \div (2) \times 100$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*per cent*
1	10.96	12.93	1.97	2.22	10.71	−0.25	−2.3
2	30.00	31.08	1.08	0.82	30.26	+0.26	+0.9
3	12.33	13.58	1.25	2.14	11.44	−0.89	−7.0
4	13.13	14.13	1.00	2.05	12.08	−1.05	−8.0
5	12.33	13.72	1.39	2.12	11.60	−0.73	−5.9
6	13.13	14.69	1.56	1.96	12.73	−0.40	−3.0
7	13.2	15.1	1.9	1.93	13.17	−0.03	−0.3
8	13.2	15.4	2.2	1.91	13.49	+0.29	+2.2
9	12.4	13.9	1.5	2.08	11.82	−0.58	−4.7
10	12.4	14.2	1.8	2.04	12.16	−0.24	−1.9
11	11.5	14.4	2.9	2.02	12.38	+0.88	+7.7
12	11.4	13.5	2.1	2.13	11.37	−0.03	−0.3
13	11.4	13.6	2.2	2.12	11.48	+0.08	+0.7
14	7.44	10.04	2.60	2.86	7.18	−0.26	−3.5
15	10.54	12.87	2.33	2.26	10.61	+0.07	+0.7
16	10.54	12.45	1.91	2.32	10.13	−0.41	−3.9
17	22.87	24.47	1.60	1.08	23.39	+0.52	+2.3
18	7.32	10.32	3.00	2.76	7.56	+0.24	+3.3
19	15.08	17.35	2.27	1.67	15.65	+0.57	+3.8
20	22.87	24.30	1.43	1.10	23.20	+0.33	+1.4
21	7.32	10.16	2.84	2.84	7.32	0.00	0.0
Average..........							±3.1

When the result of the analysis of Sample 19 is calculated with the approximate $AgIO_3$ solubility of 0.12 mM per liter found above, the correction is 2.40 mM per liter of undiluted urine, and the final chloride calculated is 14.95 mM per liter compared with 15.08 mM per liter by the Van Slyke method. However, as has been shown above, the accuracy obtained with the use of the average solubility corrections of Fig. 2 will suffice.

When the concentration of chloride in the urine was increased by 100 mM per liter, by the addition of NaCl, gasometric analysis of the 20-fold diluted urine supernatant liquid gave 113.5 mM per liter of chloride or 98.6 per cent of the amount present (K = 0.990 for albuminous urines). This was further proof that neither the bile nor the protein was a disturbing factor when chloride was high enough to eliminate the possibility of any appreciable solubility of $AgIO_3$.

Serum[19]

Measurement of Amount of Iodate Dissolved per Mole of Chloride Present, When Serum Is Diluted with 0.085 M H_3PO_4 Solution and Shaken with $AgIO_3$—In Table XVI are summarized the results of analyses of 177 sera from normal, nephrotic, and nephritic individuals. For reaction with $AgIO_3$ the serum was diluted 10- to 25-fold with 0.085 M H_3PO_4, *without removal of the proteins.* Each serum or plasma was analyzed in duplicate, two portions of the diluted serum being shaken separately with $AgIO_3$. The total dissolved iodate was determined in some of the supernatant solutions by the gasometric method described in this paper, in others by the titrimetric method outlined in Paper II (Sendroy, 1937, *a*).

For comparison the chlorides were determined by triplicate analyses, according to the nitric acid digestion method of Van Slyke (1923-24).

The comparative results by the two methods are indicated in Table XVI by the ratio $100[IO_3]:[Cl]$. In this ratio $[IO_3]$ represents the moles of iodate per liter of serum found in solution after the reaction with $AgIO_3$,[20] while [Cl] represents moles of chloride per liter of serum found by the digestion method.

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[19] Part of the work presented in the following pages was done on serum, part on plasma. Since the results were identical with both, the term serum only will, for brevity, be used.

[20] Although theoretical considerations indicate a difference of 0.2 per

The average $[IO_3]:[Cl]$ ratio of 0.985 was obtained. There was no observable difference in either the average or of the variation of these K values between normal and pathological sera. The K value of 0.985 is incorporated in Table IV in the factors for calculation of gasometric serum chlorides. Consequently, in using these factors one automatically corrects for the fact that under the conditions of the analysis the ratio $[IO_3]:[Cl]$, or K, is 0.985 rather than 1.00.

TABLE XVI

Summary of Measurements of Ratio $[IO_3]:[Cl]$*, in Analyses of Serum, without Removal of Proteins*

Iodates were determined in serum diluted 10 to 25 times in 0.085 M H_3PO_4 and shaken with $AgIO_3$. Parallel chloride determinations, directly on serum, were made by Van Slyke's (1923-24) nitric acid digestion method. The ratio $100[IO_3]:[Cl]$ indicates moles of total dissolved iodate found per 100 moles of chloride found by the digestion method.

Species	No. of sera analyzed*	Ratio $\frac{100[IO_3]}{[Cl]}$			Procedure by dissolved i was determ
		Average	Mean deviation from average	Extreme range	
Human	31	98.5	±0.41	97.5-99.3	Gasomet
	25	98.4	±0.59	97.4-99.6	Titrimet
Horse	59	98.5	±0.47	97.5-99.6	Gasomet
	10	98.6	±0.30	98.1-99.2	Titrimet
Ox	18	98.2	±0.35	97.6-99.1	Gasomet
	4	98.3	±0.20	98.1-98.5	Titrimet
Sheep	10	98.1	±0.50	97.3-99.2	"
Dog	9	98.8	±0.39	98.2-99.3	Gasomet
Rabbit	2	98.2	0.00	97.8-97.8	"
	4	98.3	±0.20	98.1-98.5	Titrimet
Mixed	5	98.6	±0.22	98.4-99.1	Gasome
Total	124	98.5	±0.41	97.5-99.6	Gasome
	53	98.4	±0.46	97.3-99.6	Titrime

* All analyses were in duplicate. The iodate determinations were made by shaking separate portions of serum or plasma with $AgIO_3$.

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cent between the results by 10-fold and by 25-fold dilution (Table VI), the difference is too small to be definitely demonstrable in serum analyses. All dilutions are, therefore, grouped together in Table XVI.

The accuracy of the value of 0.985 for K has been checked by other experiments in which the [Cl] factor of the ratio was determined simultaneously by both the nitric acid digestion and by the Sunderman-Williams (1933) alkaline digestion method (Table XVII). The two digestion methods were found to yield practically identical results with serum. As will be shown later (Tables XIX and XX), the accuracy of the ratio factor K = 0.985 has also been checked by experiments in which the [Cl] was fixed at a known value by adding known amounts of NaCl to serum which had been previously freed of chloride by dialysis.

TABLE XVII

Results of Comparison of Chloride Analyses in Plasma or Serum by Two Digestion Methods and by Gasometric and Titrimetric (Sendroy, 1937, a) Procedures of the Iodate Method

Each value given represents an individual plasma or serum sample.

Sample	Digestion methods, 3 cc. samples		Author's method, 0.5 cc. sample diluted to 10 cc. with 0.085 M H_3PO_4			
	Van Slyke (1923-24) analysis of [Cl]	Sunderman and Williams (1933) analysis of [Cl]	Gasometric procedure, 1 cc. supernatant, gas read at 0.5 cc. volume		Titrimetric procedure, 3 cc. supernatant, titrated with 0.03 N $Na_2S_2O_3$	
			Analysis of $[IO_3]$	Ratio* $\frac{100[IO_3]}{[Cl]}$	Analysis of $[IO_3]$	Ratio* $\frac{100[IO_3]}{[Cl]}$
	mM per l.	mM per l.	mM per l.		mM per l.	
Human plasma	106.0	106.5	104.8		103.9	
	106.0	106.2	104.7	98.7	104.0	98.0
Horse serum	93.0	93.0	91.8		91.6	
	93.5	93.3	92.2	98.7	91.7	98.4
Rabbit serum	102.7	102.8	101.2		101.0	
	103.2	103.2	100.9	98.3	101.0	98.2
Ox plasma	104.7	104.8	103.3		103.1	
	104.8	105.3	103.0	98.4	103.0	98.3

* The [Cl] used in the ratio is the mean of those found by the two digestion methods.

Reducing Effect of Dilute Serum on Dissolved Iodate from the Reaction of $AgIO_3$ with Chloride—As in the case of urine samples (Fig. 3), it was found that when chloride was precipitated with $AgIO_3$ from serum diluted with 0.085 M H_3PO_4, as the supernatant material was analyzed from time to time after centrifugation, there was a loss of iodate (Group A, Fig. 4). Although the values for $[IO_3]:[Cl]$ ratios found were lower for serum ($K = 0.985$) than for urine (nephritic, $K = 0.990$; normal, $K = 0.998$), the rate of disappearance of iodate after the first analysis was not so marked as for urine (Fig. 3). From the curves of Group A of Fig. 4 it is apparent that *if supernatant samples in the chloride analysis of serum are analyzed within at least 3 hours after centrifugation, no serious error from loss of iodate will occur.*

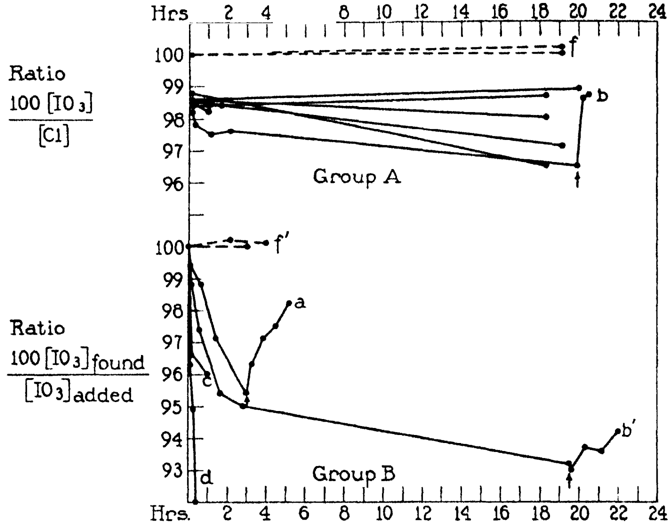

FIG. 4. Disappearance with time of dissolved iodate in diluted serum or deproteinized filtrates. Group A: iodate found from reaction of $AgIO_3$ and chloride. Supernatant fluid standing over the centrifuged solid AgCl and $AgIO_3$. Dotted lines, known chloride added to serum tungstic acid filtrates (samples *f*); solid lines, chloride precipitated from 0.085 M H_3PO_4 dilutions of serum. Sample *b* was dialyzed, chloride-free serum, with known chloride added. At the point indicated by the arrow, the tube of sample *b* was again shaken up and recentrifuged, with addition of a little more fresh $AgIO_3$. Group B: iodate found when added as KIO_3 or $KH(IO_3)_2$. Dotted lines, known iodate added to serum tungstic acid filtrates (samples *f′*); solid lines, known iodate added to 0.085 M H_3PO_4 dilutions of serum. Samples *a* and *b′* were 0.085 M H_3PO_4 dilutions of dialyzed, chloride-free sera. At the points indicated by arrows, $AgIO_3$ was added, and the samples were shaken up and centrifuged. Samples *c* and *d* were 0.085 M H_3PO_4 dilutions of normal sera.

Reducing Effect of Dilute Serum on Dissolved Iodate from Added KIO_3—From the fact that serum diluted with H_3PO_4 solution and shaken with $AgIO_3$ yielded on subsequent analysis only 0.985 mole of total dissolved iodate per mole of chloride originally present, instead of the 0.996 to 1.000 mole calculated theoretically or the 0.998 to 1.000 mole shown by pure NaCl solutions (Table XI), it appeared that a possible explanation was a slight immediate destruction of dissolved iodate by reducing agents in the serum. To test this possibility, KIO_3 in known amounts equivalent to the chloride found in normal serum was added to various samples of serum diluted with 0.085 M H_3PO_4. No $AgIO_3$ was added. The curves *c* and *d* of Group B (Fig. 4) show that the immediate destruction or loss of iodate in these sera was greater than when the iodate was derived from reaction of $AgIO_3$ with chloride. Curves *a* and *b′* were from dialyzed, chloride-free horse serum diluted with 0.085 M H_3PO_4 solution to 20 times the original serum volume. The initial concentration of IO_3 in these two samples, 5 mM, was approximately that of normal serum diluted 20-fold and shaken with $AgIO_3$ under the routine conditions of the author's method. The decrease in iodate in 3 hours, for samples *a* and *b′* amounted to about 5 per cent. For samples *c* and *d* the decrease was much greater than for *a* and *b′*, presumably on account of the presence of reducing substances which were dialyzed out of, and absent from samples *a* and *b′*.

Effect of $AgIO_3$ on the Partial Replacement of Lost Iodate—At the points marked by arrows in Fig. 4, $AgIO_3$ was added to samples *a* and *b′* to simulate (in the absence of AgCl, of course) the conditions in the usual serum chloride analysis. The mixture was shaken 2 minutes and centrifuged as in the routine analysis, and the iodate was determined again in the supernatant fluid. The fluid left after removal of the aliquot for analysis was mixed again with the same $AgIO_3$, shaken for 10 minutes, and analysis of the supernatant liquid was repeated. This procedure was again twice repeated, with shaking for 30 minutes.

Fig. 4 shows that this treatment restored the iodate of the supernatant solution of sample *a* to 98.2 per cent of that originally added. This is about the usual percentage of the theoretically expected iodate from chloride, found in acidified serum after reaction of its chloride with $AgIO_3$. Table VI shows that, with the concentration of KIO_3 present in solution, physical solution of

$AgIO_3$ could increase dissolved iodate by only 0.16 per cent of its concentration, whereas the increase in *a* was 2.8 per cent. The addition of $AgIO_3$ to sample *b′*, the iodate concentration of which had fallen to 93.0 per cent of the original after 19½ hours, caused an increase of only 1.2 per cent.

The same effect of $AgIO_3$ was demonstrated in the chloride analysis of sample *b* of Group A (Fig. 4). This dialyzed, chloride-free serum was the same as that of sample *b′* with known chloride, instead of iodate, added. After treatment with $AgIO_3$ and centrifugation, iodate analyses were made at intervals, as above. Upon being reshaken with the precipitated AgCl and $AgIO_3$ mixtures (time marked by arrow), after 20 hours the iodate was restored approximately to its original concentration.

When known iodate was added to Folin-Wu filtrates of serum, or when iodate was liberated from $AgIO_3$ by chloride in such filtrates, there was no destruction or loss of iodate, such as that found for undeproteinized serum.

Probable Reactions Which Result from Shaking Dilute Acidified Serum with Silver Iodate—From the results of Fig. 4 the following appears to be the possible sequence of events:

The chloride immediately reacts (to 99.6 to 99.8 per cent of completion) with $AgIO_3$, according to Equation 16. There are then present in the mixture solid $AgIO_3$ and AgCl, dissolved iodate and proteins, and other dissolved serum constituents which do not appear to influence the reactions considered.

There then begins a sharp reduction of the dissolved iodate to iodide. This reduction appears to be caused by the proteins, or by substances which are removable with them when the proteins are precipitated; for the disappearance of iodate does not occur in protein-free serum filtrate (samples *f* and *f′*).

However, the iodate reduced is partly replaced, because the iodide formed reacts at once with the excess $AgIO_3$ present, precipitating the I^- and dissolving IO_3^- which replaces that destroyed. The probable reactions are:

$$NaIO_3 + \text{protein (or other reductant)} \rightarrow NaI \tag{17}$$

$$NaI + AgIO_3 \rightarrow \downarrow AgI + NaIO_3 \tag{18}$$

If the reactions of Equations 17 and 18 were complete, the ratio $[IO_3]:[Cl]$ would be maintained at its theoretical level of 0.996

for 10-fold diluted serum. However, it appears either that not all of the destroyed iodate is reduced completely to iodide, or else that the reaction of Equation 18 is not complete; for the observed $[IO_3]:[Cl]$ ratio in 10- or 20-fold dilute serum is not 0.996, but only 0.985. Indeed, with reference to the reaction of Equation 18, it has already been noted that the ratio $[IO_3]:[\text{halide}]$ in 0.085 M H_3PO_4 solutions is always less when *iodide* is present than when all of the halide is chloride. Thus, when iodide was 5 per cent of the total halide (Table XIII), the ratio $[IO_3]:[I + Cl]$ was 0.985, of the same order of magnitude as that found for serum analyses.

The "protein" present in nephritic urine may cause the reactions of Equations 17 and 18 to occur in analyses of this material also, although to a less extent, because even in the most albuminous urines protein is not so concentrated as in serum. The $[IO_3]:[Cl]$ ratios observed in nephritic urines lie between the 0.985 met in serum analyses and the theoretical 0.998 obtained in analyses of salt solutions and normal urines.

Measurement of Iodate Dissolved per Mole of Serum Chloride, for Deproteinized Serum Filtrates Shaken with $AgIO_3$—Results with two types of serum filtrates are summarized in Table XVIII. In calculation of the $[IO_3]:[Cl]$ ratios the $[Cl]$ values used were the chloride concentrations per liter of serum obtained by nitric acid digestion of serum (Van Slyke, 1923–24). The $[IO_3]$ values used were the total dissolved iodate concentrations found per liter of serum in the analysis of 1:10 serum filtrates shaken with $AgIO_3$.

For the high $[IO_3]:[Cl]$ ratios averaging 1.017, thus obtained when the iodate method was applied to the tungstic acid filtrate (Folin and Wu, 1919), the probable explanation appears to be that the volume of fluid in the filtrate is really slightly less than the 10-fold serum volume assumed in calculating the $[IO_3]$ values. The 10-fold volume to which serum is diluted in the Folin-Wu tungstic acid precipitation includes not only the liquid phase, but also the solid matter of the precipitate. If the protein tungstate precipitate occupies 0.02 of the volume of the precipitate-fluid mixture, concentrations of serum crystalloids found by analysis of the *filtrate* would, when multiplied by 10 in the customary manner to estimate concentrations in serum, give values for the latter equal to 102 per cent of the correct figures. Any adsorption of a crystalloid by the precipitate would decrease the 2 per cent plus error. It appears probable from the results that the protein tungstate precipitate does not adsorb Cl^- ion, but that the zinc-protein precipitates formed in alkaline medium do adsorb it slightly. Analyses of the zinc filtrates indicate that the chloride in the zinc precipitation mixtures is approximately equally distributed, per volume unit, in the liquid and solid phases of the mixture, since the customary calculation of serum chloride values on this assumption gives correct results ($K = 0.995$).

An effect of volume displacement of fluid by protein precipitate, similar to that noted here in the tungstic acid precipitation was observed by Van Slyke, Hiller, and Berthelsen (1927) in a comparison of serum total base values, determined by ashing directly measured serum samples, with values determined by ashing 1:10 filtrates obtained by deproteinizing with trichloroacetic acid. The filtrates gave total base concentrations which, when multiplied by 10, were 3 to 4 per cent higher than those yielded by ashing directly measured serum. Similar effects of volume displacement on serum values calculated from trichloroacetic acid filtrate analyses have been reported by Ball and Sadusk (1936), who called attention to the lack of evidence for volume displacement "on other than trichloroacetic acid filtrates or in which sodium was not involved in the determination." As a matter of fact, careful inspection of Wilson and Ball's (1928) results reveals that in at least one-half of their comparisons of various methods the Whitehorn tungstic acid filtrates gave the highest results, up to a 1.5 per cent increase.

An effect of 2 per cent is more than one would estimate from the volume that would be occupied by serum protein in the dry form. This would be only about 5 per cent of the undiluted serum, or 0.5 per cent of the 10-fold diluted precipitation mixture. It appears that the precipitates as formed may hold bound or adsorbed water in amount equal to or greater than the weight of the dry proteins, and that this water may be withdrawn from the liquid phase, in so far as solvent action is concerned.

The results of Table XVIII were obtained after the possibility of effects, on analysis of deproteinized filtrates, of pH higher than those of 0.085 M H_3PO_4 solutions had been studied. Addition of phosphoric acid sufficient to make the pH 2.8 did not change the results obtained with 1:10 serum tungstic acid filtrates. On the other hand, when zinc filtrates were not acidified, the results were higher than those obtained by the digestion method. Acidification, as outlined, was therefore adopted as a preliminary procedure before the addition of $AgIO_3$ to zinc filtrates.

TABLE XVIII

Experiments with Protein-Free Serum Filtrates

Amounts of iodate dissolved by reaction of filtrates with $AgIO_3$ are compared with chloride determined directly in serum by the nitric acid digestion method of Van Slyke (1923-24).

Type of filtrate	Species of serum	No. of sera analyzed	Ratio $\frac{100[IO_3]}{[Cl]}$			Procedure by which $[IO_3]$ was determined
			Average	Mean deviation from average	Extreme range	
Folin-Wu (1919), sodium tungstate + sulfuric acid (Van Slyke and Hawkins, 1928)	Horse	15	101.7	±0.50	101.0-102.4	Gasometric
		1	101.1			Titrimetric
	Human	16	102.0	±0.77	100.9-103.1	Gasometric
		3	102.2	±0.50	101.4-102.9	Titrimetric
	Ox	2	101.5			Gasometric
		1	101.6			Titrimetric
	Rabbit	1	101.7			Gasometric
		1	100.8			Titrimetric
	Total	34	101.7	±0.64	100.9-103.1	Gasometric
		6	101.7	±0.54	100.8-102.9	Titrimetric
Somogyi (1930), zinc sulfate + sodium hydroxide. Added H_3PO_4 to filtrate	Horse	1	99.5			Gasometric
		1	100.0			Titrimetric
	Human	1	99.3			"
	Ox	1	99.7			"
	Rabbit	1	98.9			"
	Total	5	99.5	±0.32	98.9-100.0	
Letonoff (1934), zinc borate. Added H_3PO_4 to filtrate	Ox	1	99.0			Titrimetric

Experiments in Which Known Amounts of NaCl or KIO_3 Were Dissolved in Dialyzed Serum—Serum protein solutions free from chloride were obtained by dialysis in collodion sacks against repeated changes of distilled water, until no test for chloride was obtained on a deproteinized sample of the serum within the sacks. The addition of a small amount of phosphoric acid to the dialyzed

TABLE XIX

Analyses of Dialyzed, Chloride-Free Sera, with Known Amounts of KIO_3 or NaCl Added, Analyzed with and without Deproteinization

Serum dilution to 10 or 11 times, concentration of added chloride or iodate, 10 or 9 mM per liter, in each case.

Analysis No.	Composition of sample analyzed	Value found for ratio $\frac{100[IO_3]\ \text{found}}{[IO_3]\ \text{added}}$ or $\frac{100[IO_3]\ \text{found}}{[Cl]\ \text{added}}$				Theoretical value for ratio
		Ox Serum I	Ox Serum II	Horse Serum III	Ox Serum IV	
	Serum diluted directly in 0.085 M H_3PO_4					
1	1 part 0.1 M KIO_3 + 9 parts 0.085 M H_3PO_4			100.0		100.0
2	1 part 0.1 M KIO_3 + 9 parts serum diluted 10 times in 0.085 M H_3PO_4	94.9*		97.8*	97.5*	100.0
3	1 part 0.1 M KIO_3 + 9 parts serum diluted 10 times in 0.085 M H_3PO_4 + $AgIO_3$	98.2		98.3		100.0
4	1 part 0.1 M NaCl + 9 parts serum diluted 10 times in 0.085 M H_3PO_4 + $AgIO_3$	97.7		98.7	97.7	99.6
	Tungstic acid filtrate of serum					
5	1 part 0.1 M KIO_3 + 9 parts filtrate of serum	100.1		100.0	99.9	100.0
6	1 part 0.1 M KIO_3 + 9 parts filtrate of serum + $AgIO_3$	100.4		100.0		100.0
7	1 part 0.1 M NaCl + 9 parts filtrate of serum + $AgIO_3$	99.9	100.2†	100.0	99.7	99.6
8	[1 part 0.1 M NaCl + 0.9 part serum] deproteinized with tungstic acid reagent mixture, diluted to 10 parts, + $AgIO_3$		101.5‡	101.7	101.4	99.6

* First analysis only. Successive subsequent analyses of the same supernatant fluid showed a decrease of from 2 to 4 per cent in each analysis.

† Chloride content, by Whitehorn (1920-21) titration, found to give value of ratio = 100.0.

‡ Chloride content, by Whitehorn (1920-21) titration, found to give value of ratio = 101.3.

TABLE XIX—*Concluded*

Analysis No.	Composition of sample analysed	Value found for ratio $\frac{100[IO_3] \text{ found}}{[IO_3] \text{ added}}$ or $\frac{100[IO_3] \text{ found}}{[Cl] \text{ added}}$				Theoretical value for ratio
		Ox Serum I	Ox Serum II	Horse Serum III	Ox Serum IV	
	Zinc hydroxide filtrate of serum					
9	1 part 0.1 M KIO_3 + 9 parts filtrate of serum + 1 part 0.85 M H_3PO_4 + $AgIO_3$			100.0		100.0
10	1 part 0.1 M NaCl + 9 parts filtrate of serum + 1 part 0.85 M H_3PO_4 + $AgIO_3$			99.6		99.6
11	[1 part 0.1 M NaCl + 1 part serum] deproteinized with zinc sulfate and sodium hydroxide, diluted to 10 parts. 10 parts filtrate + 1 part 0.85 M H_3PO_4 + $AgIO_3$			100.5		99.6

material in the sack completely dissolved traces of precipitated protein. To the clear solution known amounts of KIO_3 or NaCl were added. The solutions were then either diluted with 0.085 M H_3PO_4 or were freed from protein by tungstic acid or zinc hydroxide precipitation. The resultant dilute solutions or filtrates were in some cases shaken with $AgIO_3$, in other cases not, as indicated in Table XIX. The dissolved iodate was determined in all. From the results in Table XIX the following conclusions may be drawn.

1. The $[IO_3]:[Cl]$ ratio of 0.985 in analyses of serum without protein removal, found when the [Cl] factor was ascertained by digestion analyses (Tables XVI and XVII), is confirmed by experiments in which the [Cl] factor was fixed by known additions of NaCl to dialyzed serum (Table XIX, Analysis 4).

2. The $[IO_3]:[Cl]$ ratios of 1.017 and 0.995 for tungstic acid and zinc hydroxide filtrates, respectively, found when serum [Cl] was determined by digestion analyses (Table XVIII), are approximated also when the chloride concentration is fixed by addition of known amounts of NaCl to dialyzed serum *before* precipitation of the proteins (Table XIX, Analyses 8[21] and 11). The probable

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[21] The results of the $AgIO_3$ method for chloride were confirmed in Analysis 8 (Ox Serum II) by determination of chloride according to the thiocyanate titration procedure of Whitehorn (1920–21).

rôles of fluid volume displacement and adsorption, by the protein precipitates, in affecting the [IO_3]: [Cl] ratios have already been discussed in connection with Table XVIII.

3. KIO_3 added to tungstic acid filtrates and to zinc hydroxide filtrates of serum *after* precipitation of the proteins is completely recovered (Table XIX, Analyses 5, 6, and 9). On the contrary, when KIO_3 is added to serum which has merely been diluted with 0.085 M H_3PO_4, without removal of the proteins, some disappearance of dissolved iodate occurs (Table XIX, Analyses 2 and 3). These results indicate that the proteins, or material capable of precipitation with them, cause a gradual disappearance of dissolved iodate from dilute, acidified serum.

This behavior offers an explanation for the fact that, when such diluted serum is shaken with $AgIO_3$, the molar ratio of dissolved iodate to chloride is only 0.985 instead of the 0.996 calculated theoretically or the 0.998 found experimentally in experiments with protein-free chloride solutions. A part of the dissolved iodate is destroyed by reaction, presumably reduction, with the proteins or other colloids.

4. When known amounts of NaCl are added to filtrates of dialyzed serum *after* precipitation of the proteins (Analyses 7[22] and 10), and the filtrates are then shaken with $AgIO_3$, values of the [IO_3]: [Cl] ratio approximating the theoretical 0.996 are obtained.

In these experiments there is no opportunity for protein precipitates to affect the results mechanically by volume displacement or adsorption, nor for dissolved proteins to destroy the dissolved iodate chemically by reduction. It appears that the only substances in serum causing the [IO_3]: [Cl] ratio to deviate from theoretical are the proteins or material which is removed with the proteins when the latter are precipitated.

Non-Effect of Serum Dilution on the [IO_3]: [*Cl*] *Ratio*—When serum was diluted with 0.085 M H_3PO_4 to from 10 to 30 times its volume, the variations in dilution caused little change in the yield of 0.985 mole of dissolved iodate per mole of chloride, as determined by digestion analysis. Tungstic acid filtrates, diluted with water to double volume after deproteinization also showed no change with dilution from the ratios (about 1.017) usually obtained in undiluted tungstic acid filtrates. From this it seems that the deviations of the $[IO_3]:[Cl]$ ratio from theoretical are unrelated to dilution *per se.* The ratio of concentrations of other serum constituents to concentration of chloride and of dissolved iodate was maintained constant in these experiments. Consequently, as dilution increased, the causative agent still remained present in the same proportion to chloride.

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[22] The results of the $AgIO_3$ method for chloride were confirmed in Analysis 7 (Ox Serum II) by determination of chloride according to the thiocyanate titration procedure of Whitehorn (1920–21).

In Table XX are the results of experiments in which the $[IO_3]:[Cl]$ ratio was determined under various conditions. In Series I are simple dilution experiments following the regularly prescribed technique for the author's method. In this series the ratio of chloride to other serum constituents remained constant, whether the dilution of serum was 10- or 30-fold. The tendency of this series was to give a fairly constant $[IO_3]:[Cl]$ ratio throughout the several dilutions.

In Series II the same serum samples were again diluted, but in such a manner that NaCl or KIO_3 was added to keep the *chloride* and hence the dissolved iodate *concentration constant.* Consequently, as dilution increased, the ratio of other serum constituents to chloride decreased. In this series the tendency was toward a decrease in deviation from the theoretical, as the dilution of serum was increased. Comparison of the same samples at the same dilutions of serum, of Series I and II, showed a decrease in deviation from theoretical as chloride or iodate was increased.

These results suggest that some constituent or constituents of serum depress the $[IO_3]:[Cl]$ ratio below the theoretical, and that the depression is proportional to the concentration of such constituents present per mole of chloride.

Effects of Various Factors on Serum Chloride Analyses by the Iodate Method. Temperature—At 15° and 35° the results of chloride analyses of serum were the same, showing that variations in room temperature, as in the case of salt solutions, have no significant effect.

Solubilities of Silver Iodate in Diluted Serum—In serum diluted to 10 times its volume with 0.085 M H_3PO_4 the solubility of $AgIO_3$ was the same as in 0.085 M H_3PO_4 alone. Hence, the fact that the $[IO_3]:[Cl]$ ratio is lower in analyses of dilute, protein-containing serum than in analyses of NaCl solutions is not caused by an effect of the proteins on $AgIO_3$ solubility.

Solubility of Air in Diluted Serum—Air solubility was found to

TABLE XX

Experiments to Determine Effect of Dilution in 0.085 M H_3PO_4 on Yield of Serum Chloride Analyses, without Deproteinization

Treatment and composition of sample — Ratio $\frac{100[IO_3]}{[Cl]}$ or $\frac{100[IO_3]}{[IO_3]\text{ added}}$*

Series I. Serum, and chloride or iodate, diluted together

Original serum dilution in 0.085 M H_3PO_4	10	15	20	30
NaCl or KIO_3, approximate mM concentration in diluted serum	10.0	6.7	5.0	3.3
1. Mixed serum + $AgIO_3$	98.4		98.7	98.6
	−1.2		−1.0	−1.3
2. Dialyzed horse serum + NaCl + $AgIO_3$	98.5		97.9	98.1
	−1.1		−1.8	−1.8
3. Ox serum + $AgIO_3$	98.4	98.0		98.2
	−1.2	−1.7		−1.7
4. " " + KIO_3 (*no* $AgIO_3$)	96.8	96.7		97.2
	−3.2	−3.0		−2.7

Series II. Serum diluted, and chloride or iodate concentration kept *constant*

Original serum dilution in 0.085 M H_3PO_4	10	15	20	30
NaCl or KIO_3, approximate mM concentration in diluted serum	10.0	10.0	10.0	10.0
1. Mixed serum + NaCl + $AgIO_3$	98.4		98.7	99.1
	−1.2		−0.9	−0.5
2. Dialyzed horse serum + NaCl + $AgIO_3$	98.5		98.5	98.5
	−1.1		−1.1	−1.1
3. Ox serum + NaCl + $AgIO_3$	98.4	98.6		99.0
	−1.2	−1.0		−0.6
4. " " + KIO_3 (*no* $AgIO_3$)	96.8	98.5		99.1
	−3.2	−1.5		−0.9

* Deviation from the theoretical for each dilution, as given in Table VI, is indicated directly below the ratio value. The values of [Cl] in the sera were determined by the Van Slyke digestion method, and are the values of [Cl] in the $[IO_3]:[Cl]$ ratios in Series I, Samples 1 and 3. In Series I and Series II, Sample 2, [Cl] values in the ratio $[IO_3]:[Cl]$ are those of NaCl added to dialyzed serum. In Series II, Samples 1 and 3, values of [Cl] are calculated from serum chloride + added NaCl. In Series I and Series II, Sample 4, values of $[IO_3]$ added are calculated from KIO_3 added to serum. In all analyses, values of $[IO_3]$ are from determined gasometric iodate measurements.

be the same, within the limits of error of analysis, in serum diluted with 0.085 M H_3PO_4, and in 0.085 M H_3PO_4 alone. Hence, the use of 0.085 M H_3PO_4 for gasometric blank analyses involves no error.

Non-Effect of Lipid—Sera from patients with marked lipemia gave $[IO_3]:[Cl]$ ratios (determined as in the experiments of Table XVI) like those of normal sera. The likelihood that fats affect the yields of iodate from chloride in serum was further diminished by the results of the addition of 5 per cent by volume of olive oil to sodium chloride solutions. Analysis of the thoroughly mixed suspension showed that the oil was entirely inert, with regard to both the solubility of solid silver iodate and the amount of iodate liberated by reaction with chloride. The theoretical amount of iodate, from the reaction of $AgIO_3$ and chloride, was found in the separated water phase.

Effect of Hemoglobin on the $[IO_3]:[Cl]$ *Ratio*—The presence in serum of red blood cells, intact or laked, in amounts up to 5 cc. of cells per 100 cc. of serum has no effect on the results of chloride analyses carried out by the iodate method without deproteinization. The chloride values found agreed exactly with those by the Van Slyke digestion method, when the K factors of serum were used in calculating the results of the iodate method. Cells do have a slight reducing effect on iodate, so that the cell concentration in ordinary whole blood diminishes the ratio $[IO_3]:[Cl]$ by about 1 per cent below the ratio found for serum. Amounts of cell material that may enter serum from accidental hemolysis of blood samples, however, are not sufficient to affect chloride results by the iodate method.

Comparison of Ultramicro- and Microprocedures with the Macroprocedures for Serum Chlorides—In Table XXI are given the results of comparative analyses of serum for very small samples, 0.02 cc. to 0.2 cc., and larger 1.0 cc. samples, all analyzed directly, without deproteinization, by the iodate method. Each analysis was carried out in duplicate, by shaking separate portions of serum with $AgIO_3$. Analyses of whole blood by deproteinization have yielded results of the same order of constancy as those given in Table XVIII. It is apparent that very little sacrifice in accuracy is entailed by the use of ultramicro and micro samples, when analyzed according to the technique given in this paper.

Claudius (1922), Patterson (1928), and Rappaport (1933) have given various modifications of the "open Carius" titrimetric method applied to small samples. Claudius used 0.02 cc. samples, but gave no data for serum analyses. Patterson used 0.2 cc. with an indicated accuracy of about ±1 per cent for serum samples. Rappaport used 0.1 cc. samples and reported results of about the same order of accuracy in the analysis of five samples. So far as

TABLE XXI

Comparison of Chlorides Determined in Serum by the Macro, Micro, and Ultramicro Forms of the Iodate Method

Sample No.	Material	Macroanalysis				Micro- or ultramicroanalysis				Deviation of micro from macro results
		Sample volume	Dilution with 0.085 M H_3PO_4*	Method†	Chloride found	Sample volume	Dilution with 0.085 M H_3PO_4	Method†	Chloride found	
		cc.	*cc.*		*mM per l.*	*cc.*	*cc.*		*mM per l.*	*mM per l.*
1	Human plasma	1	+30	G.	104.1	0.02	+0.6	G.	104.4	+0.3
2	Ox serum	1	+30	"	103.5					
				T.	103.8	0.02	+0.6	T.	103.3	−0.3
3	" "	1	+30	"	103.8	0.02	+0.6	"	105.1	+1.3
4	Horse serum	0.5	To 10	G.	91.8					
				T.	91.3	0.02	+0.6	"	92.5	+0.9
5	Ox serum	1	" 30	G.	97.9					
		1	+30	"	98.4	0.05	+1.5	G.	98.5	+0.3
6	" "	1	+30	"	98.9	0.05	+1.5	"	98.4	−0.5
7	Human plasma	1	To 25	"	109.8	0.1	+2.0	"	109.2	−0.6
8	" "	1	" 10	"	103.3	0.1	+2.0	"	103.8	+0.5
		1	" 25	"	103.9	0.2	+5.0	"	103.5	−0.4
9	" "	1	" 25	"	107.5	0.2	+5.0	"	107.6	+0.1
10	" "	1	" 10	"	107.5	0.2	+5.0	"	107.6	0.0
		1	" 25	"	107.7					
11	" "	1	+10	"	105.9	0.2	+2.0	"	106.3	+0.4
						0.2	+5.0	"	107.1	+1.2
12	" "	1	To 25	"	106.6	0.2	+5.0	"	107.0	+0.4
13	" "	1	" 25	"	89.1	0.2	+5.0	"	89.3	+0.2
14	" "	1	" 25	"	102.1	0.2	+5.0	"	102.1	0.0

* By "+30" is meant that 1 volume of serum was mixed with 30 volumes of 0.085 M H_3PO_4. "To 30" indicates dilution of 1 volume of serum to 30 volumes with the H_3PO_4.

† G. = gasometric analysis of present paper; T. = titrimetric analysis of following paper (Sendroy, 1937, *a*).

the author is aware, the present method for plasma chloride determinations is the only one available for which there has been demonstrated the degree of accuracy of the results of Table XXI for samples of 0.02 cc. and 0.05 cc.

Whole Blood

Results with Protein-Free Filtrates[23]—As for serum, the results were gaged for accuracy by comparison with results obtained by the Van Slyke (1923–24) digestion method and by the Sunderman and Williams (1933) procedure. The latter was accepted as standard; it gave whole blood chloride values averaging 0.5 per cent higher than the Van Slyke simple nitric acid digestion method. Table XXII shows that *whole blood tungstic acid filtrates* (not

TABLE XXII

Summary of Results of Comparative Chloride Analyses in Human Whole Blood by the Sunderman and Williams (1933) Digestion Method, and by the Iodate Method Applied to Deproteinized Samples

Procedure		No. of analyses	Average		Extreme range of ratio values
Sample treatment	Measurement of iodate		Values for ratio $\frac{100[IO_3]}{[Cl]}$	Deviation from mean of average ratio values	
Filtrate of (tungstic acid + H_3PO_4) diluted to 25 times serum volume	Gasometric	22	100.0	±0.74	98.7–101.3
	Titrimetric	20	100.0	±0.58	98.8–101.2
Filtrate of zinc hydroxide (1:10) diluted to 1.1 times volume with H_3PO_4	Gasometric	2	98.9		98.2– 99.6
	Titrimetric	2	98.7		98.0– 99.3

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[23] When diluted cell suspension or whole blood containing the cell colloids is shaken with $AgIO_3$, the supernatant fluid contains material which reduces the dissolved iodate, so that the iodate subsequently measured is less than theoretically corresponds to the chloride present. With whole blood diluted 1:20 in 0.085 M H_3PO_4 the effect is constant enough to permit use of a correction factor (K = approximately 0.975) without much loss of accuracy but it is preferable to remove the proteins. With separated cells, removal of the proteins is necessary.

Folin-Wu preparations, but those prescribed for this method) gave $[IO_3]:[Cl]$ ratios approaching the theoretical value of 0.999 (for these concentrations of chloride) in two series, gasometric and titrimetric (Sendroy, 1937, *a*), of twenty different samples each. Tungstic acid *serum* filtrates diluted 1:10, have been shown to yield 1.017 moles of iodate per mole of serum chloride. The lower yield of these whole blood filtrates, diluted 1:25, is probably attributable to the greater reducing action of cell constituents other than proteins on iodate—an effect which approximately balances the positive "volume displacement" error.

Somogyi zinc hydroxide filtrates of whole blood were analyzed (Table XXII). While serum results (Table XVIII) indicated values close to the theoretical $[IO_3]:[Cl]$ ratio of 0.996 (for these concentrations of chloride), the yields for whole blood filtrates, prepared in the same dilutions as serum filtrates, were slightly lower, averaging 0.988. Because of the scarcity of these data, the slight discrepancy in serum and whole blood filtrate values cannot be stated to be significant.

SUMMARY

In the proposed method chloride-containing solutions are shaken with silver iodate, whereby the Cl^- in solution is replaced by an equimolar amount of IO_3^-. The IO_3^- is then determined by its oxidative reaction with alkaline hydrazine, $2IO_3^- + 3N_2H_4 = \uparrow 3N_2 + 6H_2O + 2I^-$, with manometric measurement of the N_2 in the Van Slyke-Neill apparatus. Since IO_3^- as an oxidizing agent is hexavalent, 1 equivalent of Cl^- is represented by 6 equivalents of oxidized hydrazine. This multiplication gives the iodate method a unique advantage for microanalyses: it is possible to determine, with an error not exceeding 1 per cent, the chloride in as little as 0.02 cc. of serum. Moreover, the measurements are directly proportional to the amount of chloride present.

The method is rapid; only 2 minutes shaking are required for the reaction between $AgIO_3$ and Cl^-, while the reaction with hydrazine is instantaneous. The procedure also has the advantages of the manometric technique in freedom from standard solutions and in speed and accuracy of micromeasurement. No removal of proteins, either by precipitation or digestion, from urine, or plasma

or serum, is required, although protein-free filtrates of serum or of whole blood may also be used for analysis.

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BIBLIOGRAPHY

Ball, E. G., and Sadusk, J. F., Jr., *J. Biol. Chem.*, **113**, 661 (1936).
Brown, A. S., and MacInnes, D. A., *J. Am. Chem. Soc.*, **57**, 489 (1935).
Claudius, D., *Verhandl. 34 Kong. deutsch. Ges. inn. Med.*, Wiesbaden, 138 (1922).
Eisenman, A. J., *J. Biol. Chem.*, **82**, 411 (1929).
Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
Harvey, S. C., *Arch. Int. Med.*, **6**, 12 (1910).
Haslewood, S. A. D., and King, E. J., *Biochem. J.*, **30**, 903 (1936).
Isaacs, M. L., *J. Biol. Chem.*, **53**, 17 (1922).
Landolt-Börnstein-Roth physikalish-chemische Tabellen, Berlin, 4th edition, 456 (1912).
Letonoff, T. V., *J. Lab. and Clin. Med.*, **20**, 1293 (1934).
Patterson, J., *Biochem. J.*, **22**, 788 (1928).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Rappaport, F., *Klin. Woch.*, **12**, 1774 (1933).
Riegler, E., *Z. anal. Chem.*, **40**, 633 (1901); **41**, 17 (1902).
Sendroy, J., Jr., *Proc. Am. Soc. Biol. Chem.*, **8**, lxxxi (1935) (*J. Biol. Chem.*, **109** (1935)); *J. Biol. Chem.*, **120**, 405 (1937, *a*); **120**, 419 (1937, *b*); **120**, 441 (1937, *c*).
Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 463 (1934).
Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
Sunderman, F. W., and Williams, P., *J. Biol. Chem.*, **102**, 279 (1933).
Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923–24); **73**, 121 (1927).
Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **79**, 739 (1928).
Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, **74**, 659 (1927).
Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107**, 661 (1934).
Whitehorn, J. C., *J. Biol. Chem.*, **45**, 449 (1920–21).
Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, **79**, 221 (1928).

MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

II. TITRIMETRIC ANALYSIS

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In Paper I of this series (Sendroy, 1937, *a*) a system of chloride analysis based on the reaction

$$\text{(1)}\qquad \underset{\text{soluble}}{NaCl} + \underset{\text{insoluble}}{AgIO_3} \rightleftarrows \underset{\text{insoluble}}{AgCl} + \underset{\text{soluble}}{NaIO_3}$$

was outlined in detail, with gasometric measurement of the iodate resulting from the reaction. In this paper titrimetric measurement of the iodate is described.

Titrimetric measurement of the iodate is directly applicable to the supernatant fluid in the $AgIO_3$ precipitation of chloride from *salt solutions*, *urine*, *serum*, and *protein-free filtrate* of serum and of whole blood.[1] For the titrimetric analysis of *blood* only protein-free filtrates may be used.

The measurement is carried out according to the classical reaction for iodate in acid solution in the presence of an excess of potassium iodide. The equation applicable to the present measurements is

$$\text{(2)}\qquad KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4$$

The iodine evolved from the reaction of the iodate with acidified KI in excess is titrated against sodium thiosulfate with starch as indicator. Since 6 equivalents of iodine result from 1 of chloride, this reaction is 6 times as sensitive as the one on which the iodometric titration procedure of McLean and Van Slyke (1915), or that of Conway (1935), is based.[2]

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[1] The term "serum" is used here, as in the previous paper, for both plasma and serum.

What has been said of the advantages of the method in its gasometric form applies equally well to the titrimetric form, except that all titrimetric measurements must be compared against a known, standard iodate solution. In accuracy, convenience, and rapidity, the method in its titrimetric form is little different from the gasometric. Here also, samples of serum as small as 0.02 cc. may be used, with no loss of accuracy. By the reaction of Equation 2 about 3.2 cc. of 0.003 N $Na_2S_2O_3$ are required for titration of the iodine in the supernatant fluid available from such a sample of serum. The sensitivity of the starch-iodine end-point makes titrations easily possible to within 0.01 cc.

DESCRIPTIVE

Reagents—

Approximately 0.85 M, 0.34 M, 0.17 M, and 0.085 M Phosphoric Acid Solutions.

M/15 Potassium Acid Phosphate Solution.

Caprylic Alcohol.

Silver Iodate, Powder, C.P.

Tungstic Acid Reagent (Folin-Wu, 1919).

Zinc Hydroxide Reagent (Somogyi, 1930).

The preparation of the above reagents is described in the corresponding section of Paper I (Sendroy, 1937, *a*). In addition, there are the following:

5 Per Cent Potassium Iodide Solution—This is freshly prepared every day. 5 gm. of KI are dissolved in 100 cc. of water.

2 Per Cent Starch Solution—Of Lintner's soluble starch, 2 gm. are ground into suspension with a little water, then boiled in 100 cc. of saturated NaCl solution for 5 or 10 minutes. This reagent keeps indefinitely.

0.5 Per Cent Starch Solution—The 2 per cent solution is diluted 4 times with saturated NaCl solution.

Approximately 0.12 M (= 0.12 N) Sodium Thiosulfate Solution—About 30 gm. of $Na_2S_2O_3 \cdot 5H_2O$ are diluted to 1 liter with water.

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[2] As far as the writer is aware, there has been no previous application of this reaction to the analysis of chlorides. See the foot-note to the title page of Paper I (Sendroy, 1937, *a*).

This stock solution and the weaker solutions made from it by simple dilution are kept in the dark, in amber bottles. If aged properly, they will become very stable. Without any precautions against CO_2 we have kept even 0.001 N solution unchanged for 5 days.

Approximately 0.03 N, 0.0075 N, and 0.003 N sodium thiosulfate solutions are made from the 0.12 N solution by approximate dilution with water, to 4, 16, and 40 times volume, respectively.

0.05 M (= 0.6 N) Standard Potassium Biiodate Solution—Exactly 19.498 gm. of $KH(IO_3)_2$ are dissolved in water to make 1 liter of solution. This standard stock solution keeps indefinitely.

Standard 0.03 N and 0.0075 N potassium biiodate solutions are made from the 0.6 N solution by dilution with *0.085 M H_3PO_4 solution* to 20 and 80 times volume, respectively.

Procedure

The general procedure with titrimetric analysis of the iodate consists of the same steps described in the "Procedure" of Paper I. For the treatment of samples up to the point where the solid $AgIO_3$ and AgCl are separated from the supernatant fluid, the reader is referred to the details in the corresponding section of that paper (Sendroy, 1937, *a*) from steps (1) through (4) to the beginning of the description of the "Gasometric determination of total dissolved iodate."

Titrimetric Measurement

As in the gasometric measurement, the volume of the supernatant sample analyzed will depend on the size of the initial sample of original material and the extent of the dilution. Supernatant samples of from 0.5 cc. to 3.0 cc. volume are pipetted into a small test-tube (15 × 125 mm.).[3] 0.085 M H_3PO_4 is added to bring the volume to 3.0 cc. or 1.5 cc. Freshly prepared 5 per cent potassium iodide solution, in suitable amounts, is added and allowed to react for at least 30 seconds. The liberated iodine is then titrated with sodium thiosulfate solution. When the yellow color has been almost completely discharged, the starch indicator solution is added and the titration is carried on to an almost completely colorless, permanent end-point. The use of a constant light source, such as a daylight lamp, is desirable for this titration.

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[3] Wherever the use of a cotton plug is indicated for withdrawal of samples of the supernatant fluid in the gasometric procedure, such a filter should be used in the corresponding procedure of the titrimetric analysis.

The thiosulfate solution used is in every case of a strength such that from 2.5 to 3.2 cc. are required for complete titration of the sample. The thiosulfate is delivered, drop by drop, from a Bang 3 cc. microburette (Peters and Van Slyke (1932) p. 13) calibrated in 0.01 cc. or 0.02 cc. intervals and tipped with a pointed capillary delivering 100 to 150 drops per cc. Increments of reagent of the order of magnitude of 0.002 cc. may thus be accurately delivered.

The *standardization* of the thiosulfate is carried out by titration of an equivalent amount of the standard iodate solution in place of the supernatant sample, in *exactly* the same way as described above. No blank analysis is made in the titrimetric procedure. It is necessary only to ascertain that the acid solution used to dilute the original sample is chloride-free, and that it develops no blue color with starch when potassium iodide is added to it.

In the analysis of urine samples the yellow color of the original sample does not interfere with the preliminary iodine titration before the addition of starch. The extent of dilution with phosphoric acid, for dilute or for concentrated samples, is sufficient to produce an almost colorless solution. In the analysis of serum samples the same holds true. Upon the addition of the KI to the supernatant fluid of undeproteinized serum samples, the iodine will be observed to separate out, as if attached to protein particles. As the titration proceeds, however, this clears up, and the solution finally becomes almost entirely clear and transparent.

Specific directions for chloride determination in various kinds of materials, with titrimetric analysis of the iodate, are given below. For details of the steps leading up to the actual titrimetric analysis of the supernatant samples, the reader is referred to the corresponding sections in Paper I (Sendroy, 1937, *a*).

A. Determination of Chloride in Inorganic Material—Samples are diluted as described in the corresponding Section A of Paper I. Approximately 0.1 M neutral salt solutions or 0.1 N HCl solutions are diluted 10 to 30 times with 0.085 M H_3PO_4, then analyzed as described for serum analyses below, except that no caprylic alcohol is necessary.

B. Determination of Chloride in Urine—As for gasometric analysis, samples may be analyzed according to (*a*) a routine method used for all samples, or (*b*) a more accurate method.

Routine Method—The urine is diluted 20-fold with 0.17 M H_3PO_4, as described in the corresponding Section B of Paper I. Samples of 0.5 cc. (from very concentrated, normal urines) to 3.0 cc. (from low chloride urines) of the supernatant fluid are taken, and 0.085 M H_3PO_4 is added when needed to make the volume 3.0 cc. The analysis is completed as outlined in the previous section headed "Titrimetric measurement" by the addition of 1.0 cc. of 5 per cent KI, 1 drop of 2 per cent starch solution, and 0.03 N $Na_2S_2O_3$ delivered from a 3 cc. microburette. For the standardization, 2 cc. of 0.03 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 are titrated with 0.03 N $Na_2S_2O_3$ in exactly the same way.

The remarks made in Paper I concerning the accuracy of the routine method for urine apply equally well to the titrimetric analysis. The correction for $AgIO_3$ solubility in the case of low chloride samples is also used for titrimetric analyses. However, the use of thiosulfate of a single strength (0.03 N) for all samples necessarily limits the accuracy of the measurements in the case of low chloride urines. The resulting errors are, nevertheless, much less than those obtained by the usual Volhard titration by difference (Sendroy, 1937, *b*), especially when protein is present. For analysis by 20-fold dilution the titration with 0.03 N $Na_2S_2O_3$ will range from 0.3 cc. for a 3 cc. sample of the supernatant fluid from a urine 10 mM in chloride to about 2.4 cc. for a 1 cc. sample of supernatant fluid from a urine 240 mM in chloride.

Precise Method—Dilution with 0.17 M H_3PO_4 is made as described in the corresponding Section B of Paper I (Sendroy (1937, *a*) Table I). Samples of 2 cc. of the supernatant fluid are diluted with 1 cc. of 0.085 M H_3PO_4 and 1 cc. of 5 per cent KI solution. Titration is carried out with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point. Standardization is carried out as for the *routine* procedure.

C. Determination of Chloride in Plasma or Serum, without Deproteinization—The method of preparing samples, from 1.00 cc. down to 0.02 cc., by dilution with 0.085 M H_3PO_4, is the same as that described in the corresponding Section C for the gasometric determination (Sendroy, 1937, *a*). The use of samples of various volumes and titration with $Na_2S_2O_3$ of different, suitable concentrations make possible the maintenance of a high degree of accuracy even for 0.02 cc. samples (Sendroy (1937, *a*) Table XXI).

Macrodetermination—A sample of 0.5 cc. of serum is diluted 20 times and prepared for iodate analysis, as in the gasometric method. Of the supernatant fluid, 3 cc. are taken, usually without the necessity of a cotton plug in the pipette. Then 1 cc. of 5 per cent KI is added, and the titration is carried out with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point. For the thiosulfate standardization 2 cc. of 0.03 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 1 cc. of 5 per cent KI are titrated with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point.

Microdetermination—Samples of 0.2 cc. or 0.1 cc. of serum are diluted and prepared for iodate analysis, as in the gasometric method. Of the supernatant fluid, samples of 1 cc. are taken with a pipette equipped with a cotton plug. Then 2 cc. of 0.085 M H_3PO_4 and 0.3 cc. of 5 per cent KI are added. The titration is carried out with 0.0075 N $Na_2S_2O_3$, 1 drop of 0.5 per cent starch solution being added near the end-point. For the thiosulfate standardization 2 cc. of 0.0075 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 0.3 cc. of 5 per cent KI are used.

Ultramicrodetermination—Samples of 0.05 cc. or 0.02 cc. are diluted and prepared for iodate analysis, as in the gasometric method. *For the 0.05 cc. analysis,* the titration of a 1 cc. sample of the supernatant fluid is carried out exactly as above for *micro* samples.

For the 0.02 cc. analysis, the sample of supernatant fluid is withdrawn in a 0.5 cc. pipette, calibrated between marks. A cotton plug filter is used in the pipette. To the sample are added 1.0 cc. of 0.085 M H_3PO_4 and 0.1 cc. of 5 per cent KI. The titration is carried out with 0.003 N $Na_2S_2O_3$, 1 drop of 0.2 per cent starch solution being added near the end-point. For the thiosulfate standardization 1 cc. of 0.0075 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 0.1 cc. of 5 per cent KI are used.

D. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—These filtrates are prepared for analysis as described in the corresponding Section D (Sendroy, 1937, *a*) on gasometric analysis.

(*a*) *Tungstic acid filtrates of plasma or serum.* When enough filtrate is available, samples of 1.5 cc. or 1.0 cc. of the supernatant fluid are taken in a pipette without a cotton plug. There are then added 1.5 or 2.0 cc. of 0.085 M H_3PO_4, respectively, followed by 1.0 cc. of 5 per cent KI. Titration is carried out with 0.03 N $Na_2S_2O_3$ and 1 drop of 2 per cent starch solution. Standardization is carried out with 2 cc. of 0.03 N $KH(IO_3)_2$, as described above for the *macro*determination without deproteinization.

If there is a scarcity of filtrate, samples of 0.5 cc. of the supernatant fluid, taken in a pipette equipped with a cotton plug, may be used. There are added 1.5 cc. of 0.085 M H_3PO_4, 0.3 cc. of 5 per cent KI, and 0.0075 N $Na_2S_2O_3$. Near the end-point 1 drop of 0.5 per cent starch is added. Standardization is carried out with 2 cc. of 0.0075 N $KH(IO_3)_2$, as described above for the *micro*determination without deproteinization.

(*b*) *Tungstic acid filtrates of whole blood* are made, and prepared for analysis as described for the gasometric method. The usual Folin-Wu filtrate may not be used. Of the supernatant fluid from the *macro* (1 cc.) or the *micro* (0.2 cc.) determination, 1 cc. samples are taken. There are added 2.0 cc. of 0.085 M H_3PO_4 and 0.3 cc. of 5 per cent KI. Titration is carried out with 0.0075 N $Na_2S_2O_3$, standardized as described above for the *micro*determination without deproteinization.

(*c*) *Zinc hydroxide filtrates of plasma or serum* are made, and prepared for analysis as described for the gasometric method. Samples of 1.5, 1.0, or 0.5 cc. of the supernatant fluid are analyzed as described above (*a*) for tungstic acid filtrates of serum.

Calculations

From theoretical considerations (Sendroy, 1937, *a*) and experimental results, 1 mole of iodate found by analysis corresponds to $1/K$ mole of chloride originally present. In the titration 1 mole of iodate is equivalent to 6 moles of thiosulfate. Hence, the general equation for the calculation of titrimetric results is:

$$[\mathrm{Cl}]_i = v \times \frac{n \times d \times 1000}{6 \times K \times s} \tag{3}$$

where

$[\mathrm{Cl}]_i$ = mM chloride per liter of original sample

v = cc. volume of thiosulfate used for titration of the supernatant sample

TABLE I

Factors for Calculation of Titrimetric Chloride Analyses by Iodate Method

The chloride concentration of the original samples is found by multiplying the product (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$ by the factor f, according to Equation 4, $[Cl] = v \times n \times f$.

Material	Analysis	Original sample volume	d = dilution factor of original sample in supernatant liquid	s = volume of supernatant liquid analyzed	Approximate normality of $Na_2S_2O_3$ used	K = ratio $\frac{[IO_3]}{[Cl]}$	f = factor by which to multiply (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$, to obtain		
							mM Cl per liter	Gm. NaCl per liter	Mg. NaCl per 100 cc.
		cc.		*cc.*					
Normal urine	Routine	0.5	20	0.5	0.03	0.998	6,680*	390.8*	
				1.0	0.03	0.998	3,340	195.4	
				3.0	0.03	0.998	1,113	65.1	
Albuminous urine	Routine	0.5	20	0.5	0.03	0.990	6,734*	393.9*	
				1.0	0.03	0.990	3,367	197.0	
				3.0	0.03	0.990	1,122	65.6	
Serum, without deproteinization	Macro	0.5	20	3.0	0.03	0.985	1,128		6,599
	Micro	0.2	26.05	1.0	0.0075	0.985	4,408		25,787
		0.1	31.05	1.0	0.0075	0.985	5,254		30,736
	Ultramicro	0.05	31.05	1.0	0.0075	0.985	5,254		30,736
		0.02	31.15	0.5	0.003	0.985	10,540		61,659
Serum, tungstic acid filtrate			10	1.5	0.03	1.017	1,093		6,394
			10	1.0	0.03	1.017	1,639		9,588
			10	0.5	0.0075	1.017	3,278		19,176
Whole blood, tungstic acid filtrate	Macro	1.0	25	1.0	0.0075	1.000	4,167		24,377
	Micro	0.2	25	1.0	0.0075	1.000	4,167		24,377

Serum, zinc hydroxide filtrate			11†	1.5	0.03	0.995	1,228		7,184
			11†	1.0	0.03	0.995	1,843		10,782
			11†	0.5	0.0075	0.995	3,685		21,557

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* *If urine analyzed by the routine method is calculated to contain less than 60* mM *of chloride per liter (or 3.51 gm. of NaCl per liter), correction for* $AgIO_3$ *solubility must be made according to Fig. 2 of the previous paper (Sendroy (1937, a) p. 363).*

† If the serum filtrate + H_3PO_4 mixture is prepared by diluting 9 volumes of filtrate to 10 volumes (see foot-note 13 (Sendroy, 1937, *a*)), d = 11.11 and the factors f are multiplied by 1.010.

$n =$ standardized normality of the thiosulfate
$d =$ dilution factor for the number of times original sample was diluted, with H_3PO_4 or protein precipitant, before shaking with $AgIO_3$
$s =$ cc. volume of supernatant sample used for titration
$K = \frac{[IO_3]}{[Cl]} =$ empirical reaction yield factor expressing the ratio of the concentration of iodate, found by analysis, to the concentration of chloride originally present, per liter of original sample

For convenience in calculating, Equation 3 can take the form

$$[Cl] = v \times n \times \left(\frac{d \times 1000}{6 \times K \times s}\right) = v \times n \times f \tag{4}$$

When results in terms of gm. of NaCl per liter, or mg. of NaCl per 100 cc. are desired, the values of f for mM of Cl per liter are multiplied by 0.0585, or 5.85, respectively.

In Table I are given values for the factor f, under different conditions of analysis. When the product (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$ is multiplied by f, the result in terms of mM of chloride per liter, gm. of NaCl per liter, or mg. of NaCl per 100 cc. is found.

Example—A sample of serum was diluted to 20 times the original volume (0.5 to 10 cc.) before addition of silver iodate. Of the centrifuged supernatant liquid, 3 cc. were then analyzed, the titration requiring 2.97 cc. of the "0.03 N" $Na_2S_2O_3$, the standardized normality of which was found to be 0.03039 N. (A 2 cc. sample of exactly 0.03 N $KH(IO_3)_2$ required 1.974 cc. of the $Na_2S_2O_3$ at the end-point.) From Equation 3 the chloride content of the original sample was calculated to be

$$\frac{2.97 \times 0.03039 \times 20 \times 1000}{6 \times 0.985 \times 3} = 101.8 \text{ mM per liter}$$

Calculated according to Equation 4 and Table I, the result was found to be $2.97 \times 0.03039 \times 1128 = 101.8$ mM of chloride per liter, or $2.97 \times 0.03039 \times 6599 = 595.5$ mg. of NaCl per 100 cc.

Correction for Solubility of $AgIO_3$ in the Routine Method for Urine—In the *routine* analysis of urine, when the calculation according to Table I gives a result less than 60 mM of chloride per liter (or 3.51 gm. of NaCl per liter), a solubility correction for

$AgIO_3$ must be applied, exactly as for the gasometric method, according to Fig. 2 of Paper I (Sendroy (1937, *a*) p. 363).

However, in the routine analysis of such low chloride urines, *when the titration is less than 1.8 cc.*, instead of calculating according to Table I, and subtracting the correction for $AgIO_3$ solubility, we may calculate the corrected chloride concentration graphically, directly from the volume of $Na_2S_2O_3$ used for the titration. Thus, if Equation 4 be recast for this purpose,

TABLE II

Table for Direct Graphical Calculation of Titrimetric Results for the Routine Analysis of Low Chloride Urines

For supernatant samples of 3 cc. titrated with approximately 0.03 N $Na_2S_2O_3$, the titration volume, *v*, of the approximately 0.03 N $Na_2S_2O_3$ actually used for the titration, must first be corrected to the corresponding volume, *v'*, of *exactly* 0.03 N $Na_2S_2O_3$, by multiplying by the standardization factor, *n*/0.03.

v' = volume of exactly 0.03 N $Na_2S_2O_3$ used	Chloride concentration of original sample corrected for solubility of $AgIO_3$ in the supernatant	
	mM Cl per liter	Gm. NaCl per liter
1.789	59.76	3.497
1.491	49.62	2.903
1.193	39.44	2.307
0.895	29.16	1 706
0.596	18.62	1.089
0.537	16.42	0.961
0.477	14.20	0.831
0.418	11.92	0.697
0.358	9.62	0.563
0.298	7.14	0.418
0 239	4.39	0 257
0.209	2.86	0.167

$$[\mathrm{Cl}]' = (v \times n \times f) - S \tag{5}$$

where $[\mathrm{Cl}]' = [\mathrm{Cl}] - S$ = the concentration of chloride in mM per liter, in the original sample, corrected for the solubility of $AgIO_3$ in the supernatant liquid, and S = the variable solubility correction from Fig. 2 of the preceding paper (Sendroy, 1937, *a*).

Then we may write for the routine urine analysis of 3 cc. samples of supernatant liquid, using the average K value of 0.994,

$$[\mathrm{Cl}]' = (v' \times 33.53) - S \tag{6}$$

where $v' = v \times (n/0.03)$ = volume v of approximately 0.03 N $Na_2S_2O_3$ used for titration, corrected to a corresponding volume of *exactly* 0.03 N $Na_2S_2O_3$.

Table II, calculated from Equation 6, may be used to draw curves with $[\mathrm{Cl}]'$ and v' as coordinate axes for the direct reading of corrected chloride concentrations from titration volumes of exactly 0.03 N $Na_2S_2O_3$. It is desirable that the graphs be drawn on a fairly large scale, so that there may be no great loss of accuracy in the readings of low chloride concentrations.

Examples—Two samples of urine, Samples A and B, were analyzed by the *routine* method. The supernatant samples of 3 cc. required 2.50 cc. and 0.481 cc., respectively, of 0.03 N $Na_2S_2O_3$, the standardization factor of which was 0.992. The analysis of Sample A was calculated according to Table I to be 2.50 × 0.02976 × 65.1 = 4.84 gm. of NaCl per liter. The analysis of Sample B was calculated according to a chart based on Table II, whereby 0.481 cc. × 0.992 = 0.477 cc. was found to correspond to a value of 0.831 gm. of NaCl per liter. Calculated according to Table I, Sample B showed 0.481 × 0.02976 × 65.1 = 0.932 gm. of NaCl per liter. With the correction for $AgIO_3$ solubility from Curve B, Fig. 2, of Paper I, the final result was 0.932 − 0.104 = 0.828 gm. of NaCl per liter.

The theoretical aspects of the reaction of Equation 1, whereby chloride is precipitated by $AgIO_3$, have already been discussed in Paper I (Sendroy, 1937, *a*). The experimental results obtained by titration have been included there and require no further discussion here.

SUMMARY

The present paper contains an outline of a titrimetric procedure for the measurement of the iodate in the chloride method outlined in the preceding paper. The IO_3^- is determined by its reaction with acidified KI, with starch as an end-point indicator: $KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4$. Thus, as in the gasometric method, the hexavalent oxidative power of IO_3^- results in 1 equivalent of Cl^- being represented by 6 equivalents of free iodine.

The accuracy and rapidity of this procedure are about the same as

of the gasometric procedure. Here, likewise, no removal of proteins, either by precipitation or digestion, from urine, or plasma or serum, is required, although protein-free filtrates of serum and of whole blood may be used for analysis.

BIBLIOGRAPHY

Conway, E. J., *Biochem. J.*, **29**, 2221 (1935).
Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, **21**, 361 (1915).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335 (1937, *a*); **120**, 441 (1937, *b*).
Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).

MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

III. COLORIMETRIC ANALYSIS

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In this paper colorimetric measurement of the iodate from $AgIO_3$ in the author's method of chloride determination (Sendroy, 1937, *a*, *b*) is described. The reaction involved is the same as that of the titrimetric measurement; namely,

$$KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4 \quad (1)$$

Instead of a thiosulfate titration of the liberated iodine, however, the color of the latter is compared in a colorimeter with that of a known, standard solution of iodate, treated exactly as is the supernatant solution of the unknown from the chloride precipitation.

Colorimetric measurement of the iodate is applicable to the supernatant fluid from the $AgIO_3$ precipitation of chloride from *salt solutions* and *protein-free filtrates* (Folin and Wu, 1919; Somogyi, 1930) of plasma and of whole blood. It is not generally suitable for urine analysis, since proteins, when present, interfere.

The percentage accuracy of the colorimetric method is not equal to that of the gasometric or titrimetric methods, but the colorimetric technique can be used for the measurement of exceedingly small amounts of chloride, when the physical limitations of the other methods render them inadequate for this purpose; and the average error of the colorimetric procedure, ±1.5 per cent, permits its use for many purposes. Samples representing 0.0006 mg. of NaCl may be used for the iodate colorimetric reading, whereas at least 80 times as much is the required minimum for gasometric or titrimetric measurement.

Colorimetric methods of chloride measurement have been described by Isaacs (1922) and by Conway (1935). In Isaacs' procedure the addition of solid silver chromate causes the precipitation of the chloride and the passing into solution of chromate ion which is measured colorimetrically against suitable standards. For serum analysis Folin-Wu (1919) filtrates are used. There have been several modifications of this method, the most refined form of which is that developed by Westfall, Findley, and Richards (1934). By treating the chromate filtrate with sym-diphenylcarbazide (Cazeneuve's reagent), they obtained an increase in color intensity of 100 times. The serum analysis was confined to the use of zinc hydroxide filtrates, since the tungstic acid in Folin-Wu filtrates reacts with the Cazeneuve's reagent. Westfall, Findley, and Richards were able to analyze 0.3 c.mm. samples of serum, containing 0.01 mg. of NaCl, with an estimated average error of less than 3 per cent.

Conway's procedure involves the wet combustion of chloride in an especial apparatus, with absorption of the volatile chlorine in KI solution. In microanalyses the iodine set free is measured colorimetrically. This may be done by the use of spectral color filters for the yellow iodine color or by direct readings of the blue color developed upon the addition of starch. Conway used the latter technique for analyses at the lower limit of his method, whereby samples containing 0.01 mg. of NaCl were analyzed for chloride with an estimated average error of 6 to 7 per cent. However, he apparently did not apply the colorimetric procedure to blood and serum analysis.

In the procedure to be described the yellow iodine color may be read directly, with or without filters. However, the author prefers the color reading of the blue developed upon addition of starch. A readable yellow color is obtained for the iodine from an iodate solution diluted to 0.5 mM. By the reaction of Equation 1 the iodine obtained per mole of chloride is 6 times that obtained in Conway's method. With a further 50-fold dilution, and addition of starch, a very intense blue color is obtained which can be read even more easily than the yellow. Since the solutions used for the blue color reading represent a dilution of 1:10,000 of a 0.1 M chloride solution, and since microcolorimetric readings may be made with 1 cc. samples of solution, one is limited only by the accuracy with which small volumes of solutions and reagents may be measured and handled (Westfall, Findley, and Richards, 1934). However, for practical purposes 0.1 cc. of serum or 0.2 cc. of 1:10 serum filtrate as starting material may be considered the lower limit of sample volumes to which the colorimetric method may be applied in serum chloride analysis. Serum filtrates of either tungstic acid or of zinc hydroxide may be used. For whole blood the especial (tungstic acid + H_3PO_4) filtrate (Sendroy, 1937, *a*, *b*) is used. The colorimetric results for serum and whole blood agree with the gasometric and titrimetric values within extreme limits of ±3 per cent, with an average discrepancy of less than ±1.5 per cent.

DESCRIPTIVE

Reagents—

The preparation of the following reagents is described in the corresponding section of Paper I (Sendroy, 1937, *a*):

Approximately 0.85 M, 0.34 M, 0.17 M, 0.085 M, 0.017 M Phosphoric Acid Solution.

M/15 Potassium Acid Phosphate Solution.

Caprylic Alcohol.

Silver Iodate, Powder, C.P.

Tungstic Acid Reagent (Folin-Wu, 1919).

Zinc Hydroxide Reagent (Somogyi, 1930).

The preparation of the following reagents is described in the corresponding section of Paper II (Sendroy, 1937, *b*):

5 Per Cent Potassium Iodide Solution.

1 per cent potassium iodide solution is made from the 5 per cent solution by dilution with water.

2 Per Cent Starch Solution—While this reagent, made as described, keeps indefinitely, it is best to prepare it anew every 3 weeks when used for colorimetric work, since age seems to affect the quality of the color.

0.05 M Standard Potassium Biiodate Solution.

Standard 0.005 M, 0.001 M, and 0.0002 M solutions are made from the 0.05 M solution by dilution with water, to 10, 50, and 250 times volume, respectively.

Procedure

The general procedure, with colorimetric analysis of iodate, consists of the same steps described in the "Procedure" of Paper I on gasometric measurement (Sendroy, 1937, *a*). For the treatment of samples up to the point where the solid $AgIO_3$ and AgCl are separated from the supernatant fluid, the reader is referred to the details in the corresponding section of that paper, from steps (1) through (4), to the beginning of the description of the "Gasometric determination of total dissolved iodate."

Colorimetric Measurement

As for the gasometric and titrimetric procedures, the original sample is diluted so that the total dissolved iodate in the supernatant liquid after addition of $AgIO_3$ is between 3 and 12 mM. The volume of the supernatant fluid taken for analysis then depends on that concentration and on the amount of supernatant fluid available. Samples of from 0.02 to 0.5 cc. volume of the latter are pipetted into a suitable volumetric flask of volume such that the concentration of the iodate in the final diluted supernatant fluid approximates either 0.5 or 0.01 mM for the yellow or blue color reading, respectively. There are then added H_3PO_4 and KI solution, and the yellow iodine color is allowed to develop.

For the *yellow color reading*, dilution is then made with water to the volume mark. For the *blue color reading*, water is first added to the flask to about 0.9 volume. The capacity of the flask should be 50 times that of the flask used when the yellow color is read. Starch solution is added *slowly, with mixing*, and then water to the volume mark. The color developed is compared, in each case, with that of a simultaneously prepared standard. When blue color readings are made over a period of several hours, the inconvenience of making a new standard for each determination may in some cases be avoided by the use of one standard solution (not more than 24 hours old) with a correction for color change on standing.

The *standard* is prepared from another portion of equal volume of the same sample of the unknown which has been diluted with H_3PO_4 (in the case of salt solutions), or with protein precipitant (in the case of serum and blood filtrates), but to which *no* $AgIO_3$ has been added. Standard iodate solution is added, then the other reagents, exactly as for the analysis of the unknown.

The inclusion of the unknown solution or filtrate in the standard is necessary for accurate work, because the color developed (especially the blue with starch) is affected by the composition of the solution. With these standards, the compositions of standard and unknown color solutions are made more nearly alike. The former will, however, contain a portion of chloride salt lacking from the latter.

It is not necessary to prepare several standards of different strengths. One, of the constant iodate composition corresponding to that of a 0.1 M solution diluted 200 to 10,000 times, for the yellow or blue color, respectively, is sufficient. An approximate color match is obtained by diluting the unknown sample of supernatant fluid so that the final iodate concentration approximates that of the standard within ±20 per cent. For samples of uncertain chloride concentration, the correct dilution must first be approximated by preliminary trial and error dilutions (by varying the size of the supernatant fluid sample used), to match the color of a simple iodate standard, containing *none* of the diluted or deproteinized unknown. For samples of which the chloride concentration is approximately known, as for the filtrates of serum or of whole blood, dilution of the supernatant fluid is always made in the same way (to 20 or 1000 times volume for serum filtrates, and to 6⅔ or 333⅓ times volume for whole blood filtrates). As has already been mentioned, *in all cases exact matching of the color requires that the salts and acid in the unknown supernatant fluid and in the standard be qualitatively and quantitatively as nearly the same as possible.*

Depending on the size of the colorimeter cups used, from 1.0 to 5.0 cc. of the final solution (the diluted supernatant fluid) are needed for a determination, and a similar amount for the standard. The author has used a Bausch and Lomb colorimeter, with constant artificial light source. The standard solution is set at 25 mm. on one side, say on the right, and compared with itself on the other side, the left. The unknown is then substituted on the left side. Five or six readings of each solution against the (right) standard should be sufficient and within the range of 0.3 mm.

Specific directions for chloride determination in salt solutions, and in serum and whole blood filtrates, with colorimetric analysis of the iodate, are given below and outlined in Table I. For details of the steps leading up to the colorimetric analysis of the supernatant samples, the reader is referred to the corresponding sections in the gasometric paper (Sendroy, 1937, *a*).

A. Determination of Chloride in Inorganic Material—Samples

TABLE I

Outline of Procedure for Colorimetric Analysis of Chloride by Silver Iodate Method

Sample material	Approximate chloride concentration of diluted sample or filtrate	Volume of supernatant (after $AgIO_3$)	Volume of diluted sample or filtrate (no $AgIO_3$)	Volume of standard $KH(IO_3)_2$ solution	Volume of H_3PO_4 solution	Volume of KI solution	For color development			
							Yellow	Blue		
							H_2O to final volume	H_2O to approximate volume	Volume of 2 per cent starch solution	H_2O to final volume
Concentration of reagents				0.005 M	0.085 M	5 per cent				
	mM per l.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
$AgIO_3$ dissolved in H_2O	0.2	5.0			0.4	0.2		90	1.0	100
Standard				0.5	2.0	1.0		450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	10	0.5			1.5	1.0	10	450	5.0	500
Standard			0.5	0.5	1.5	1.0	10	450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	5	1.0			1.0	1.0	10	450	5.0	500
Standard			1.0	0.5	1.0	1.0	10	450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	3	1.5			0.5	1.0	10	450	5.0	500
Standard			1.5	0.5	0.5	1.0	10	450	5.0	500
Tungstic acid serum filtrate	7-12	0.5			2.0	1.0	10	450	5.0	500
Standard			0.5	0.5	2.0	1.0	10	450	5.0	500

Tungstic acid blood filtrate	2.6–3.8	1.5			0.5	1.0	10	450	5.0	500
Standard			1.5	0.5	0.5	1.0	10	450	5.0	500
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.5			1.5	1.0	10	450	5.0	500
Standard			0.5	0.5	1.5	1.0	10	450	5.0	500
Concentration of reagents				0.001 M	0.085 M	1 per cent				
Tungstic acid serum filtrate	7–12	0.2			0.8	2.0		180	2.0	200
Standard			0.2	1.0	0.8	2.0		180	2.0	200
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.2			0.6	2.0		180	2.0	200
Standard			0.2	1.0	0.6	2.0		180	2.0	200
Concentration of reagents				0.0002 M	0.017 M	1 per cent				
Tungstic acid serum filtrate	7–12	0.02			0.4	0.2		18	0.2	20
Standard			0.02	0.5	0.4	0.2		18	0.2	20
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.02			0.3	0.2		18	0.2	20
Standard			0.02	0.5	0.3	0.2		18	0.2	20

are diluted as described in the corresponding Section A of Paper I on the gasometric analysis. When approximately 0.1 M neutral salt solutions are analyzed, they are diluted to 10 times with 0.085 M H_3PO_4, then analyzed exactly as described for serum filtrates below. No caprylic alcohol is necessary. When supernatant samples are 5.0 or 3.0 mM in iodate, 2 or 3 times as much sample is taken as of serum filtrate supernatant liquid (see Table I).

The solubility of $AgIO_3$ (Sendroy (1937, *a*) p. 368) can easily be determined by following the outline in Table I. Samples of 5 cc. of the saturated solution are diluted to 100 cc. and compared with the 0.01 mM iodate standard, prepared as indicated. If the solubility is to be determined in a solvent other than water, the same concentration of the same solvent must be present in the standard solution.

B. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—These filtrates are prepared for analysis as described in the corresponding Section D of Paper I (Sendroy, 1937, *a*) on gasometric analysis.

(*a*) *Tungstic acid filtrates of plasma or serum.* When enough filtrate is available,[1] samples of 0.5 cc. of the supernatant liquid are taken in a pipette, with a cotton plug if necessary, and transferred to either a 10 cc. or a 500 cc. volumetric flask. There are then added 2.0 cc. of 0.085 M H_3PO_4 solution, followed by 1.0 cc. of 5 per cent KI solution. With the 10 cc. flask, water is added to volume, and the *yellow color* is read. With the 500 cc. flask, the solution volume is increased to 90 per cent of the capacity by addition of water. Then 5.0 cc. of 2 per cent starch solution are added slowly with continuous shaking. After dilution to the mark with water, the *blue color* is read. For the *standard,* in place of the supernatant fluid, 0.5 cc. of the serum filtrate (not treated with $AgIO_3$) and 0.5 cc. of 0.005 M $KH(IO_3)_2$ are treated as above.

Analyses of 0.2 cc. and 0.02 cc. samples of supernatant liquid are outlined, together with the above, in Table I.

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[1] When tungstic acid filtrates of serum are prepared solely for use in the colorimetric chloride determination, dilution of small samples with tungstic acid reagent mixture may be made as follows: 0.5 cc. to 5.0 cc., 0.2 cc. + 2.0 cc., and 0.1 cc. + 1.0 cc. Ordinarily, the use of smaller volumes than these will not prove practicable. From the 0.5 cc. and 0.2 cc. samples, 0.5 cc. of supernatant liquid will be available for analysis, and from the 0.1 cc. samples, 0.2 cc. samples of supernatant liquid may be used.

(*b*) *Tungstic acid filtrates of whole blood* are made, and prepared for analysis as described for the gasometric method. The usual Folin-Wu filtrate may not be used. Of the supernatant fluid, 1.5 cc. are taken as sample, 0.5 cc. of 0.085 M H_3PO_4 is added, and the rest of the analysis is carried out as described for serum above. Full details are given in Table I.

(*c*) *Zinc hydroxide filtrates of plasma or serum* are made, and prepared for analysis as described for the gasometric method. Of the supernatant liquid, 0.5 cc. samples are used, with 1.5 cc. of 0.085 M H_3PO_4. The rest of the analysis is carried out as for tungstic acid filtrates, as described above. Full details for the analysis of 0.5 cc., 0.2 cc., and 0.02 cc. samples of supernatant liquid are given in Table I.

Calculations

The general equation for the calculation of the colorimetric results is

$$(2) \qquad [\mathrm{Cl}]_i = \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{m \times d \times D}{K}$$

where

$[\mathrm{Cl}]_i$ = mM chloride per liter of original sample

m = mM concentration of iodate in the final, dilute standard solution used for the reading

d = dilution factor for the number of times the original sample was diluted with 0.085 M H_3PO_4 or protein precipitant, before addition of $AgIO_3$

D = dilution factor for the number of times the supernatant sample was diluted for the development of color

$K = \frac{[\mathrm{IO_3}]}{[\mathrm{Cl}]}$ = empirical reaction yield factor expressing the ratio of the concentration of iodate found by analysis, to the concentration of chloride originally present, per liter of sample (see "Experimental" section of Paper I (Sendroy, 1937, *a*).

For convenience in calculation, Equation 2 can take the form:

$$(3) \qquad [\mathrm{Cl}] = \frac{\text{reading of standard}}{\text{reading of unknown}} \times F$$

where

$$F = \frac{m \times d \times D}{K}$$

When results in terms of gm. of NaCl per liter, or mg. of NaCl per 100 cc. are desired, the values of F for mM of Cl per liter are multiplied by 0.0585, or 5.85, respectively.

The values of F for the several analyses outlined above are given in Table II. When the blue color is read, after the value of [Cl] has thus been found, depending on that value, a correction is applied from Fig. 1. This correction is for deviations found from Beer's law when blue color readings are made, as outlined above, against the 0.005 M $KH(IO_3)_2$ standard.

TABLE II

Table of Factors for Calculation of Results of Chloride Analyses from Colorimetric Iodate Readings, According to Equations 2 and 3

Sample material	d = original sample dilution factor	K = ratio $\frac{[IO_3]}{[Cl]}$	D = supernatant sample dilution factor	For color		
				Yellow	Blue	
				$m = 0.5$	$m = 0.01$	Correction to be made from Fig. 1, when indicated by
				Values of factor F by which to multiply ratios $\frac{\text{reading standard}}{\text{reading unknown}}$ to obtain mM Cl per liter original sample		
Inorganic salt solutions		0.996–1.000		Use Equation 2		Curve 2
Tungstic acid serum filtrate	10	1.017	20	98.3		
			1000		98.3	Curve 3
	11*	1.017	20	108.2		
			1000		108.2	" 4
Tungstic acid blood filtrate	25	1.000	6⅔	83.3		
			333⅓		83.3	Curve 1
Zinc hydroxide serum filtrate + H_3PO_4	11	0.995	20	110.6		
			1000		110.6	
	11.11	0.995	20	111.7		
			1000		111.7	

* Prepared as described in foot-note 1.

Example—A sample of 0.5 cc. of the centrifuged supernatant fluid, from the treatment of a tungstic acid filtrate (1:10) of serum with $AgIO_3$, was treated with phosphoric acid and KI and diluted to 10 cc. volume (Table I). To 0.5 cc. of a sample of the same filtrate, *not* treated with $AgIO_3$, was added 0.5 cc. of a standard solution of 0.005 M $KH(IO_3)_2$. After addition of 2.0 cc. of 0.085 M H_3PO_4 and 1.0 cc. of 5 per cent KI, this also was diluted to 10 cc. volume. Of this standard yellow color solution, 5 cc. was poured into each cup of the colorimeter. The reading on the right was set at 25.0 mm. The average of the readings on the left was 24.8 mm. The standard solution on the left was then replaced by the unknown solution, the average of the readings for which was 23.4 mm. From Equation 2 the chloride concentration of the original serum sample was calculated as

$$\frac{24.8}{23.4} \times \frac{0.5 \times 10 \times 20}{1.017} = 104.2 \text{ mM Cl per liter}$$

or more simply, from Table II, as

$$\frac{24.8}{23.4} \times 98.3 = 104.2 \text{ mM Cl per liter}$$

The same procedure was repeated, but with dilution in 500 cc. flasks, with addition of 5.0 cc. of 2 per cent starch solution after water had been added to about 450 cc. When the blue color solutions were matched, the readings were found to average 24.9 and 23.8 for standard and unknown, respectively. The original sample chloride concentration was then calculated as

$$\frac{24.9}{23.8} \times \frac{0.01 \times 10 \times 1000}{1.017} = 102.8 \text{ mM Cl per liter}$$

or

$$\frac{24.9}{23.8} \times 98.3 = 102.8 \text{ mM Cl per liter}$$

From Fig. 1 (Curve 3) a correction value of 0.7 mM per liter was added to give a final corrected value of 103.5 mM of Cl per liter.

EXPERIMENTAL

The experimental work having to do with the reaction of $AgIO_3$ with halides under many different conditions has been reported in Paper I (Sendroy, 1937, *a*). Studies of the factors affecting the colorimetric determination of the iodine obtained by the reaction of Equation 1 are given in the following.

Colorimetric Chloride Determinations, with Reading of the Yellow Iodine Color—In Table III are given the results of preliminary work on colorimetric chloride determinations.[2] Values obtained by reading the yellow colors of the iodine solutions were compared against gasometric or titrimetric analyses, the accuracy of which

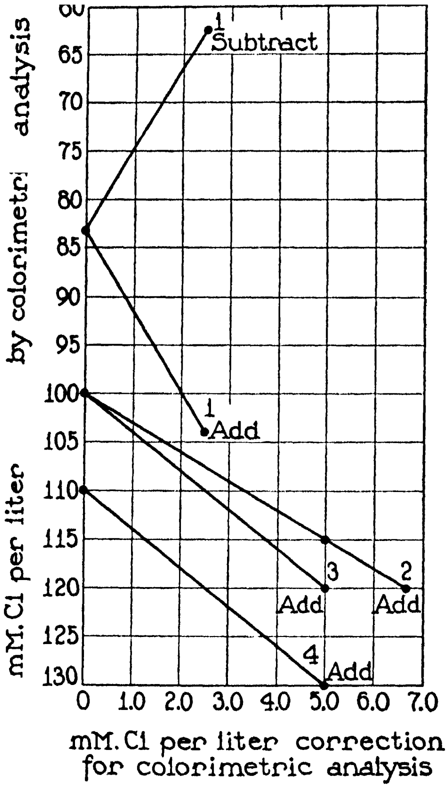

FIG. 1. Chart showing corrections to be applied to colorimetric chloride values determined by readings of the blue starch-iodine color. Curve 1, for tungstic acid + H_3PO_4 blood filtrates; Curve 2, for inorganic chloride or iodate solutions; Curve 3, for tungstic acid serum filtrates (serum diluted 1:10); Curve 4, for tungstic acid serum filtrates (serum diluted 1:11). See foot-note 3.

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[2] Table III includes one urine analysis. While the colorimetric method is satisfactory for normal urines, which are diluted so much that the yellow of the sample can be compensated for, and does not interfere, that is not the case for low chloride samples. Furthermore, if protein is present, the colorimetric method is inapplicable, without deproteinization.

has already been demonstrated (Sendroy, 1937, *a*). The results for various materials analyzed were as good as might be expected of most colorimetric procedures. To this extent they would be suitable for purposes for which no greater accuracy was required. However, yellow is not an easy color to read, and while the interposition of a blue glass filter partially overcomes this objection, it was thought desirable to develop a more sensitive color for direct reading.

TABLE III

Comparison of Colorimetric with Titrimetric and Gasometric Results by the Silver Iodate Chloride Method. Yellow Iodine Color Read

Material analyzed	d = original sample dilution factor	D = supernatant dilution factor for color reading	Chloride found		
			Gasometrically	Titrimetrically	Colorimetrically
			mM per l.	*mM per l.*	*mM per l.*
0.1 M NaCl in 0.085 M H_3PO_4	10	20			97.0
	10	20			99.4
	20	10			99.6
	25	8			100.5
	40	5			100.0
Normal urine in 0.17 M H_3PO_4	20	25		183.1	179.5
Tungstic acid serum filtrate	10	20	103.5	103.6	105.5
	10	20	102.2		104.6
	10	20	94.8	94.5	93.9
	10	20	102.5	101.7	98.2
	10	20	100.7		100.4
	10	20		105.7	110.8
	10	20	102.3		106.9
	10	20		104.3	105.7
Zinc hydroxide serum filtrate	11	20		101.2	100.7
Tungstic acid blood filtrate	20	$8\frac{1}{3}$	90.5	90.5	91.5
	25	$6\frac{2}{3}$	84.8		83.2
	11	15	82.2	82.0	82.5

Colorimetric Chloride Determinations, with Reading of the Blue Starch-Iodine Color—Although many investigators have attempted to use the blue color of the starch-iodine complex as a measure of iodine concentration in dilute solutions, their efforts have apparently met with little success. However, Turner (1930), Woodard (1934), and Conway (1935) have reported the use of starch for colorimetric iodine determinations under definite, limited conditions.

It appears from the literature and from our own experiments that so many factors affect the nature of the color produced and its sensitivity, that only under strictly controlled conditions is the starch-iodine reaction applicable to colorimetric iodate analyses. Such factors may be the concentrations of starch, of iodine, of KI, and of other salts, the degree of acidity, the presence of organic matter, and the quality of the particular starch suspension used. However, when the conditions of analysis are definitely set, and the standard and unknown prepared as alike as possible, little difficulty should be encountered. Although the authors cited above have used the starch-iodine color only in inorganic, comparatively simple solutions, the author has been able to utilize it in the presence of protein-free filtrates of serum and of whole blood, containing from 0.6 to 0.9 mg. of iodine per 100 cc. of solution.

Stability of Blue Starch-Iodine Color—In the initial experiments with iodate solution it was observed that newly prepared standards of 0.01 mм iodate (about 0.76 mg. of iodine per 100 cc.), prepared as described above, were weaker than ones which had been prepared several hours previously. This is in agreement with the observations of Turner (1930) who found that there was a slight increase in color after 16 hours. This he ascribed either to change in dispersion of the starch or to a slight oxidation of KI.

The results of our experiments in this connection are shown in Fig. 2. In five different experiments a color standard was prepared from 0.005 м $KH(IO_3)_2$ solution, as described above. In each experiment, at varying time intervals, new standards of the same strength were prepared, and their color compared with that of the first standard. The results of Fig. 2 show that, on the average, there is a general increase in color of a standard on standing for 4 hours, at the rate of about 1 per cent per hour. After 4 hours, standards have been found to be stable for about 30 hours, at least. After that time, a slight flocculation of particles has been observed.

If color comparisons against fresh standards are made within

10 minutes, the color change is imperceptible. On the other hand, if both standard and unknown are made simultaneously, and read after several hours, the color change in both will be very nearly proportional, and should not affect the results. If a large series of measurements, covering a period of several hours, is made on solutions differing in composition *only* by the amount of iodine present, one standard solution may be used. A plus correction is then made in the calculation of the unknown for increase in color of the standard, corresponding to the time elapsed between its preparation and its use against any given unknown. Thus, a simple iodate solution which matched the color of a standard prepared 4 hours previously would be calculated as being not 0.01 mM, but 0.0104 mM.

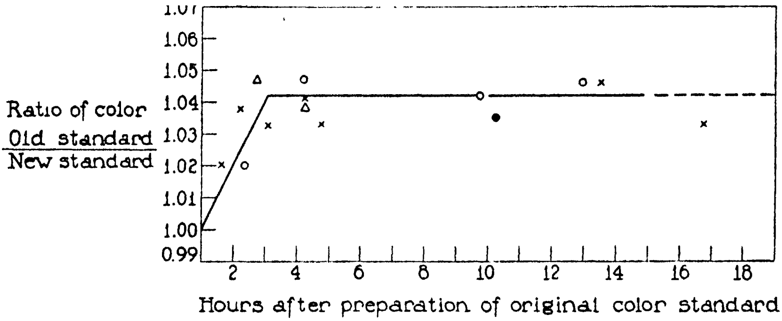

FIG. 2. The change in intensity, with time, of the blue starch-iodine color developed from standard solutions of 0.01 M iodate diluted to 1000 times volume. In each of five experiments solutions were made and the color developed anew at various intervals, to be compared with the color of the first (old) standard.

Proportionality of Color to Iodine from Inorganic Iodate Solutions and Serum and Blood Filtrates—According to Beer's law, intensity of color is directly proportional to concentration. An index of the validity of this law for any color system is the correspondence of the value for the ratio, *reading of standard*:*reading of unknown*, to that of the ratio, *concentration of unknown*:*concentration of standard*, at ratio values more or less than 1. When the one standard solution concentration of 0.01 mM iodate was used, the validity of Beer's law under the conditions of analysis described above was tested for inorganic iodate solutions and serum and blood filtrates. The deviations found were then applied as corrections for the analysis of different kinds of material.[3] The corrections are given in Fig. 1, not as deviations above and below the value of 1 for the ratio of the color readings, but in each type of analysis, for the concentrations above and below that at which the color of the material analyzed matches that of the standard. Thus from the application of Equation 2, tungstic acid blood filtrates (barring some consistent error for all determinations) match the color of the standard when the blood is 83.3 mM in chloride.

Iodate solutions of known concentrations were prepared so that they were weaker or stronger than the standard to within 20 per cent. The average results of six experiments showed that Beer's law held for solutions weaker than the standard, but not stronger. At higher concentrations the development of color was not strong enough, as is indicated by the correction Curve 2 of Fig. 1. Thus, an iodate solution of 0.125 M, diluted 10,000 times, gave a value of the ratio, *reading of standard:reading of unknown*, of 1.2 instead of 1.25.

For *serum and blood filtrates*, the criterion of deviation from Beer's law was the gasometric or titrimetric analysis of the same sample used for the colorimetric determination. The colorimetric standard used for each sample was of the same strength in iodate, 0.01 mM, and contained the appropriate filtrate as described in the procedure and outlined in Table I. *Tungstic acid filtrates of serum* (1:10) showed a minus deviation from gasometric or titrimetric results, which increased as the chloride concentration of the sample was increased, above 100 mM (Fig. 1, Curve 3). For the small samples, diluted 11-fold, the point of departure would be 110 mM, corresponding to the concentration of the original sample. The 11,000-fold dilution of such a sample would match the color of the standard 0.01 mM iodate (Fig. 1, Curve 4). *Tungstic acid* ($+H_3PO_4$) *filtrate of whole blood* showed a linear deviation, minus

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[3] Wright (1926-27) has called attention to the possible danger of taking too literally the colorimetric correction curves found in one laboratory, or by one individual. In the writer's opinion, if the directions given above are followed strictly in detail, no serious error should result in the use of the corrections given in Fig. 1. However, the analyst should satisfy himself on this point by making several comparative analyses such as those indicated in the following sections of this paper.

TABLE IV

Comparison of Colorimetric with Titrimetric or Gasometric Tungstic Acid Serum Filtrate Results by the Silver Iodate Chloride Method. Blue Starch-Iodine Color Read

Sample No.	Sample*	Chloride found by analysis				
		Titrimetric or gasometric	Colorimetric	Correction for colorimetric results from Fig. 1, Curve 3	Colorimetric corrected	Deviation of colorimetric from titrimetric or gasometric
		mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	Pig plasma	108.2	106.9	+1.7	108.6	+0.4
2	" "	110.7	106.5	+1.6	108.1	−2.6
3	" "	115.2	112.5	+3.1	115.6	+0.4
4	Ox serum†	104.3	102.5	+0.6	103.1	−1.2
5	" "	105.5	104.6	+1.1	105.7	+0.2
6	Human serum	103.8	104.3	+1.1	105.4	+1.6
7	" "	104.3	102.5	+0.6	103.1	−1.2
8	" plasma	104.2	101.2	+0.3	101.5	−2.7
9	" "	107.6	104.3	+1.1	105.4	−2.2
10	" serum	115.3	115.1	+3.8	118.9	+3.6
11	" plasma	116.6	115.1	+3.8	118.9	+2.3
12	" "	103.0	102.0	+0.5	102.5	−0.5
13	" "	96.9	98.3		98.3	+1.4
14	" "	107.4	108.4	+2.1	110.5	+3.1
15	" serum	102.4	99.5		99.5	−2.9
16	" "	103.0	101.2	+0.3	101.5	−1.5
17	" "	80.8	82.0		82.0	+1.2
18	" "	87.0	87.1		87.1	+0.1
19	" "	90.7	90.3		90.3	−0.4
20	" "	105.6	106.1	+1.5	107.6	+2.0
21	" "	87.0	86.9		86.9	−0.1
22	" "‡	87.0	86.5		86.5	−0.5
23	" "†	87.0	86.7		86.7	−0.3
24	" "†	105.6	103.7	+0.9	104.6	−1.0
Average						±1.4

Supernatant samples of 0.5 cc. were used, except where otherwise indicated.

* See foot-note 4.

† Indicates 0.02 cc. sample.

‡ Indicates 0.2 cc. sample.

above, and plus below, the critical concentration, corresponding to 83.3 mM chloride (Fig. 1, Curve 1). *Zinc hydroxide filtrates of* *serum* (+H_3PO_4) showed no consistent deviation from Beer's law in the range observed.

The colors obtained in the analysis of the serum filtrates (tungstic acid and zinc hydroxide) were a true blue, and little difficulty was experienced in matching them with that of the standards. The colors of the whole blood filtrates were slightly purplish, and sometimes did not match the standard color solutions well. Nevertheless, by making allowance for this, two analysts were able to agree well in their analyses of these samples.

TABLE V

Comparison of Colorimetric with Titrimetric Results, of Tungstic Acid (+H_3PO_4) Whole Blood Filtrate Analyses by the Silver Iodate Method. Blue Starch-Iodine Color Read

Human blood Sample No.*	Chloride found by analysis				
	Titrimetric	Colorimetric	Correction for colorimetric result from Fig. 1, Curve 1	Colorimetric corrected	Deviation of colorimetric from titrimetric
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	91.4	89.5	+0.8	90.3	−1.1
2	78.8	80.5	−0.3	80.2	+1.4
3	85.9	85.6	+0.3	85.9	0.0
4	76.9	79.9	−0.4	79.5	+2.6
5	82.2	82.8	−0.1	82.7	+0.5
6	85.3	86.2	+0.4	86.6	+1.3
7	81.5	81.8	−0.2	81.6	+0.1
8	79.3	80.6	−0.3	80.3	+1.0
9	86.1	86.3	+0.4	86.7	+0.6
10	98.7	98.3	+1.8	100.1	+1.4
11	76.5	76.9	−0.8	76.1	−0.4
12	81.2	82.5	−0.1	82.4	+1.2
13	66.1	69.1	−1.7	67.4	+1.3
14	101.9	98.3	+1.8	100.1	−1.8
15	70.2	69.5	−1.6	67.9	−2.3
16	71.7	72.1	−1.3	70.8	−0.9
Average					±1.1

Supernatant samples of 1.5 cc. were used.

* See foot-note 4.

Comparison of Colorimetric with Gasometric and Titrimetric Analyses by the Iodate Method—With the average correction curves of Fig. 1, the colorimetric results were corrected and compared with values for the same samples obtained gasometrically or

TABLE VI

Comparison of Colorimetric with Titrimetric or Gasometric Zinc Hydroxide Serum Filtrate Results by the Silver Iodate Chloride Method. Blue Starch-Iodine Color Read

Human plasma Sample No.*	Chloride found by analysis		
	Titrimetric or gasometric	Colorimetric	Deviation of colorimetric from titrimetric
	mM per l.	*mM per l.*	*mM per l.*
1	118.1	121.2	+3.1
2	115.7	115.1	−0.6
3	118.1	119.3	+1.2
4	115.7	114.0	−1.7
5	86.9	85.3	−1.6
6	93.1	89.8	−3.3
7	96.6	97.2	+0.6
8	110.1	112 8	+2.7
9	116.3	115.7	−0.6
10	104 6	106.3	+1.7
11	108.1	109.2	+1.1
12	101.2	102.3	+1.1
13	103.0	103.0	0.0
14	79.8	79.0	−0.8
15	86.2	86.7	+0.5
16	89.2	89.8	+0.6
17	105.2	104.2	−1.0
18	103.0	103.4	+0.4
19†	103 0	105.1	+2.1
20	86.2	85.7	−0.5
21†	86 2	84.4	−1.8
22‡	105.2	107.0	+1.8
Average			±1.3

Supernatant samples of 0.5 cc. were used, except where otherwise indicated.

* See foot-note 4.

† Indicates 0.2 cc. sample.

‡ Indicates 0.02 cc. sample.

titrimetrically. The average deviation of the colorimetric measurements from the gasometric or titrimetric was less than 1.5 mM per liter with extremes of 3 mM per liter in the three series shown in Tables IV, V, and VI.[4] The corrected colorimetric results are all plotted in Fig. 3, against values known, or determined by some other measurement. The straight line is the theoretical path of results following Beer's law exactly.

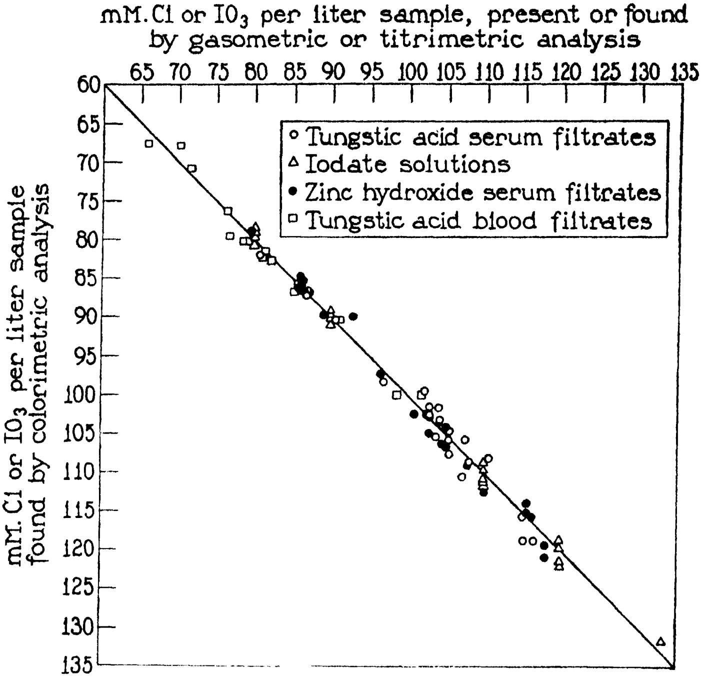

FIG. 3. Colorimetric results, corrected when so indicated in Fig. 1, are plotted against known or gasometric or titrimetric values. The straight line is a theoretical one and indicates the path of results when Beer's law is followed exactly.

From these analyses and from the evidence presented in Paper I (Sendroy, 1937, *a*) with regard to the accuracy of the gasometric and titrimetric procedures, it appears that the accuracy of the colorimetric procedure in the silver iodate method of analysis of serum and whole blood is such that the results indicate the amounts present within an average discrepancy of ±2 per cent.

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[4] The analyses of Tables IV, V, and VI are not within normal ranges. Pathological material was used, and in many cases the chloride concentration was purposely altered by addition of water or salt, to obtain extreme values.

SUMMARY

The present paper contains an outline of a colorimetric procedure for the measurement of the iodate in the chloride method outlined in Papers I and II. The IO_3^- is determined by the reaction of Paper II, with the yellow color of the free iodine, or the intense blue of the complex with starch, serving as a measure of concentration.

This procedure is not as accurate or as rapid as the gasometric or titrimetric procedure. It cannot be used in the presence of proteins, which must first be removed from urine, plasma, or whole blood. However, it is an easy procedure to follow; it requires but one standard solution and the minimum of technical ability. Furthermore, it is applicable to the analysis of extremely small samples of material.

BIBLIOGRAPHY

Conway, E. J., *Biochem. J.*, **29**, 2221 (1935).

Folin, O., and Wu, J., *J. Biol. Chem.*, **38**, 81 (1919).

Isaacs, M. L., *J. Biol. Chem.*, **53**, 17 (1922).

Sendroy, J., Jr., *J. Biol.* **120**, 335 (1937, *a*); **120**, 405 (1937, *b*).

Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).

Turner, R. G., *J. Am. Chem. Soc.*, **52**, 2768 (1930).

Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107**, 661 (1934).

Woodard, H. Q., *Ind. and Eng. Chem., Anal. Ed.*, **6**, 331 (1934).

Wright, S. L., Jr., *J. Biol. Chem.*, **71**, 209 (1926–27).

NOTE ON ERRORS IN THE ANALYSIS OF CHLORIDE IN ALBUMINOUS URINE

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With the exception of the writer's silver iodate method in its gasometric (Sendroy, 1937, *a*) or titrimetric (Sendroy, 1937, *b*) form, there appears to be no method of chloride analysis in the literature which does not require the preliminary removal of proteins when present, either by deproteinization or by wet or dry ashing, if accurate results are to be obtained. Nevertheless, in the application of some chloride methods to urine analysis, steps to insure protein removal are deliberately omitted, apparently on the assumption that urine contains no protein, or so little that it need not be removed. Such an assumption is valid for normal urines, but not for the albuminous ones which make up a large part of the urines in which chloride analyses are of clinical interest. Furthermore, it is this type of urine (*e.g.*, nephritic) which is also often of low chloride content, so that the importance of errors becomes magnified.

The present critique is limited to the following methods.

Probably the most widely used method for urine chloride is *Harvey's* (*1910*) *simple and rapid application of Volhard's* (*1878*) *method.* The excess silver is titrated with sulfocyanide immediately after the precipitation, and in the presence of the chloride.

Of late, because of their obvious simplicity, *indicator adsorption methods* (Fajans and Wolf, 1924) have attracted attention in biological work. In these methods the chloride is titrated directly with standard $AgNO_3$ in the presence of an "adsorption" indicator. At the equivalence point the slightest excess of silver ion is adsorbed by the precipitated silver chloride, as is the anion of the indicator. The two adsorbed ions form the highly colored compound the appearance of which indicates the end-point.

In *Saifer and Kornblum's* (*1936*) *procedure* the urine is acidified with H_2SO_4; then diphenylamine and $K_2Cr_2O_7$ are added. Titration is carried out with $AgNO_3$ to the end-point.

In *Collier's* (*1936*) *procedure* the urine is adjusted to pH 7.0 to 7.5, then titrated with $AgNO_3$ with, dichlorofluorescein as indicator.

In the writer's *silver iodate method* (Sendroy, 1937, *a*, *b*) the urine is acidified with H_3PO_4, more or less diluted, and solid $AgIO_3$ is added. After thorough shaking and centrifugation, the dissolved iodate in the supernatant fluid is determined gasometrically or titrimetrically. Two techniques have been developed, one carried out under the optimum conditions to insure maximum accuracy, and the other in which some accuracy is sacrificed for the sake of maximum convenience and rapidity. The two procedures, "precise" and "routine," are both applicable to normal urines and to albuminous samples without removal of proteins.

As a standard of comparison Van Slyke's (1923–24) open Carius modification of the Volhard method, with nitric acid ashing, has been used.

Analysis of Albuminous Urines by the Van Slyke (*1923–24*), *Sendroy* (*1937, a, b*), *and Volhard-Harvey* (*1910*) *Methods*—Table I gives comparative analyses of albuminous urines, of low and ordinary chloride content, by several methods. As has already been shown (Sendroy, 1937, *a*), the agreement between the nitric acid digestion method, in which the proteins are digested, and the "precise" iodate method, carried out in the presence of the proteins, is good. The results by the "routine" iodate procedure are somewhat less accurate, but lie within the limits required for most clinical work.

However, serious discrepancies were found in the analyses of Samples 1 to 6, when analyzed by the simple sulfocyanate titration method. These six samples contained considerable amounts of protein and little chloride. Whether analyzed by the Volhard-Harvey method unmodified, or modified by reducing the initial amount of $AgNO_3$ used, the results obtained by two analysts, A and B, were at times not only widely divergent, but were always considerably lower than by the other two methods.

When Samples 7 to 9 were analyzed, the results agreed with the digestion and iodate methods. These samples contained little protein and a normal amount of chloride. Apparently, under these conditions, a little protein does not affect the Volhard titration. On the other hand, the inaccuracy of the method when applied to Samples 1 to 6 is not caused entirely by protein, although the large amounts present do interfere. The error in the analysis of protein-free, *low chloride* samples by this indirect method (by difference) was demonstrable even in 15 mM pure NaCl solutions for which values of 14.0, 12.3, and 13.7 mM per liter were obtained at different times, when the analyses were carried out under the conditions adopted for urine analysis.

TABLE I

Chloride Analysis of Albuminous Urines by Van Slyke (1923–24), Sendroy (1937, a, b), and Volhard-Harvey (1910) Methods

Urine Sample No.	Proteins by analysis	Chloride concentration found by analysis according to method of							
		Van Slyke	Sendroy				Volhard-Harvey*		
			Precise gasometric	Precise titrimetric	Routine gasometric	Routine titrimetric	With 5 cc. $AgNO_3$		With 10 cc. $AgNO_3$ analyzed by B
							Analyzed by A	Analyzed by B	
	gm. per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	17.0	13.2	13.0	13.1	13.2	13.5	7.9	9.1	8.2
2	19.0	12.4	12.2	12.2	11.8	12.2	6.8	8.2	5.6
3	4.7	11.5	11.5	11.4		12.4	7.7	7.5	7.4
4	31.0	11.4	11.4	11.2	11.4	11.5	3.4	7.7	4.8
5	4.9	13.1	13.1	13.1	12.1	12.7	10.6		
6	4.6	7.44	7.49	7.52		7.18	4.28	4.62	
7	0.3	155.2	155.0	154.8			151.7		153.3
8	0.5	244.2	243.6	242.7					243.4
9	1.4	210.5	209.0	207.9					207.8

* Analyses by two individuals, A and B. Analyses with 10 cc. of $AgNO_3$ were carried out as described by Peters and Van Slyke ((1932) p. 834). Analyses with 5 cc. of $AgNO_3$ were carried out in the same way, but the use of an additional 10 cc. of NH_4CNS for the titration was thereby avoided.

Analysis of Albuminous Urines by the Adsorption Methods of Saifer and Kornblum (1936) and of Collier (1936)—Table II gives the results of analyses of two samples of albuminous urine, with varying amounts of added NaCl, carried out exactly as described by the authors of the two adsorption methods used. We were unable to obtain any end-point for albuminous urine, at any concentration of chloride, by Saifer and Kornblum's method. Nor could an end-point be obtained by Collier's method in albuminous urine with the lower concentrations of chloride. End-points were observed when the chloride concentration was increased. They were not sharp, however, and the titration led to inaccurate results, unless the chloride concentration was high.

TABLE II

Chloride Analysis of Albuminous Urines by Adsorption Methods of Saifer and Kornblum (1936) and of Collier (1936)

Urine Sample No.	Protein in urine by analysis	Determination	Composition of solution titrated			Chloride concentration calculated as if all in urine		Adsorption analysis according to
			Urine	0.1 N NaCl	H_2O	Known	Found by adsorption analysis	
	gm. per l.		*cc.*	*cc.*	*cc.*	*gm. NaCl per l.*	*gm. NaCl per l.*	
6	4.6	A	1		2	0.44[*]	No end-point	Saifer and Kornblum
		B		1	2	5.85	5.90	
		C	1	1	1	6.29	No end-point	
		D	1	2		12.14	" "	
18	48	C	1	1	1	6.28[†]	" "	
6	4.6	A	1		5	0.44[*]	No end-point	Collier
		B		1	5	5.85	5.86	
		C	1	1	4	6.29	7.33	
		D	1	2	3	12.14	12.78	
18	48	C	1	1	4	6.28[†]	No end-point	

[*] Found by Van Slyke (1923-24) analysis.

[†] 0.43 gm. per liter, found by Van Slyke (1923-24) analysis of urine alone.

Effect of Aspirin Ingestion on Chloride Analyses—As noted by Jeffrey (1927), the urine of individuals who have taken aspirin shows a red tinge which interferes with the end-point observation of the Volhard titration. He suggested a suitable color control by the use of an extra urine sample. This device enables one to carry out the determination, but the end-point is still poor, and the titration inaccurate.

In one such experiment to test this point, a subject took 15 grains of aspirin in ½ hour. Urine was collected 3 hours later, colored red. By different determinations the following values for chloride were found (concentration in mM of Cl per liter): Van Slyke digestion, 229.8 (good end-point); Volhard-Harvey, 224.8 (poor end-point); Sendroy iodate, gasometric "precise" 229.8, titrimetric "precise" 229.0 (good end-point). The digestion and iodate methods were unaffected by the urine of the subject taking aspirin.

SUMMARY

Chloride titrations in albuminous urines by the Volhard and indicator adsorption methods are inaccurate and sometimes impossible to carry out, unless the protein is removed. With typical nephritic urines, low in chloride and containing protein, these methods, including the generally used Volhard-Harvey, give grossly inaccurate results. The iodate method (Sendroy, 1937, *a*, *b*) can be applied to albuminous urine without removal of the protein.

BIBLIOGRAPHY

Collier, V., Jr., *J. Biol. Chem.*, **115**, 239 (1936).
Fajans, K., and Wolf, H., *Z. anorg. u. allg. Chem.*, **137**, 221 (1924).
Harvey, S. C., *Arch. Int. Med.*, **6**, 12 (1910).
Jeffrey, W. H., *J. Lab. and Clin. Med.*, **13**, 687 (1927).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Saifer, A., and Kornblum, M., *J. Biol. Chem.*, **114**, 551 (1936).
Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335 (1937, *a*); **120**, 419 (1937, *b*).
Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923–24).
Volhard, J., *Z. anal. Chem.*, **17**, 482 (1878).

THE VERATRINE ALKALOIDS

II. FURTHER STUDY OF THE BASIC DEGRADATION PRODUCTS OF CEVINE

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Since our recent communication[1] on the degradation of cevine with zinc dust and soda lime, additional data have been obtained, which may bear some relationship to the nature of the dicyclic tertiary base, $C_{10}H_{19}N$, there described and may ultimately prove of significance in the interpretation of the structure of cevine itself.

In following further the products of the zinc dust distillation, it was found that the tertiary base $C_7H_{15}N$ was formed in larger amount than first suspected. This was made possible by a preliminary approximate separation of the basic mixture into strong and weak bases; *i.e.*, those of the saturated and those of the pyridine series, and finally by separation of the strong base fraction into tertiary and secondary base fractions with nitrous acid. It was further found that the base $C_7H_{15}N$ is optically active and was identified as *d*-N-methyl-β-pipecoline by direct comparison with its enantiomorph. This was prepared by cleavage of *dl*-β-pipecoline by the method of Ladenburg[2] and by conversion of pure *l*-β-pipecoline into the N-methyl derivative, according to the method used by Jacobi and Merling[3] for the racemic base. The mixture of the picrate of the resulting active base with the substance obtained from cevine proved indistinguishable in properties from the picrate of *dl*-N-methyl-β-pipecoline. This procedure was found necessary, since the racemic salt had a lower melting point than either of the active salts.

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[1] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **119**, 141 (1937).

[2] Ladenburg, A., *Ber. chem. Ges.*, **27**, 75 (1894); *Ann. Chem.*, **364**, 227 (1908).

[3] Jacobi, W., and Merling, G., *Ann. Chem.*, **278**, 6 (1893).

The secondary base fraction obtained by hydrolysis of the nitroso compounds gave an appreciable amount of β-pipecoline (apparently mostly racemic) which was isolated as the hydrochloride. Its identity was confirmed by the preparation of a *3,5-dinitrobenzoyl derivative* which was compared with that obtained from synthetic β-pipecoline. No evidence of the presence of coniine in this secondary base fraction could be obtained.[4]

In view of this experience we returned to a further study of the lower boiling fraction of the mixture of hydrogenated bases which resulted from the distillation of cevine with soda lime. After separation into secondary and tertiary basic fractions, an examination of the former showed it to be a mixture. In one experiment, partly active β-pipecoline (*d* base) was obtained in appreciable amount, and its nature confirmed by the preparation of the 3,5-dinitrobenzoyl derivative. However, in another experiment an extremely small amount, relatively, of a secondary base was obtained, which was converted into the 3,5-dinitrobenzoyl derivative. Analysis indicated it to be the acyl derivative of a propylpiperidine and this seemed to be confirmed by direct comparison with the acyl derivative prepared from *d*-coniine, although the rotation indicated extensive racemization. This result may thus be regarded as partial confirmation of the earlier report of Macbeth and Robinson[4] who described, however, the isolation of the antipode *l*-coniine from cevine. However, when opportunity is found, this point will be investigated further. Unfortunately, in our hands it has seemed essential to sacrifice a very large amount of cevine for the purpose. Our experience has shown that the reaction distillate is much more complex than would appear from the report of Macbeth and Robinson, and the detection of coniine more difficult.

In the weak base fraction we have already reported the isolation of a base $C_8H_{11}N$ as a picrate which melted at 132–133°. The attempt has since been made to determine its identity by oxidation, since its properties did not correspond with those of any recorded substance. On oxidation with permanganate a dibasic acid was obtained, which melted at 270–272° with decomposition and was readily decarboxylated to nicotinic acid. This ready decarboxylation indicated an α-carboxyl group and together with recorded data on the pyridine dibasic acids pointed definitely to pyridine-2,5-dicarboxylic acid or isocinchomeronic acid. The formation of such a dibasic acid would indicate, therefore, that the base, $C_8H_{11}N$, is either 2-ethyl-5-methyl pyridine or 2-methyl-5-ethyl pyridine. However, the picrate of the latter has been described[5] as melting at 164°, thus leaving the former substance as the probable one. When opportunity presents, an attempt will be made to prepare this base synthetically for comparison with the cevine degradation product.

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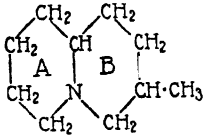
[4] Macbeth, A. K., and Robinson, R., *J. Chem. Soc.*, **121**, 1571 (1922).

A preliminary attempt can be made to interpret these observations. If the previously described dicyclic base $C_{10}H_{19}N$, which was obtained on soda lime distillation with subsequent hydrogenation, is given the formula of a methyl octahydropyridocoline (or of a dimethyl octahydropyrrocoline)



it is possible that an unsaturated precursor possessing such a ring system and formed from cevine on zinc dust distillation could give an N-methyl-β-pipecoline as well as β-pipecoline itself by cleavage of Ring A. Similarly, a pyridine base by dehydrogenation and cleavage of Ring A could give a 2-ethyl-5-methyl pyridine. Finally, the formation of β-pipecoline and possibly α-propylpiperidine (coniine) or a homologue might be similarly explained. Whether a dicyclic base of this type occurs as such in cevine and is attached by a side chain or directly fused to the remainder of the molecule can be answered only by further work.

Further investigation of the non-basic portion of the cevine molecule is now in progress.

EXPERIMENTAL

Zinc Dust Distillation of Cevine—This operation was performed essentially as previously described[1] with the exception that a rough separation of weak and strong bases was made as follows. In an experiment starting from 60 gm. of cevine about 80 cc. of additional acetic acid were added to the solution after hydrogenation and the mixture was steam-distilled into dilute hydrochloric acid. After collection of about 2 liters of distillate which carried over a mixture of weak bases and non-basic material, the residue was made alkaline with sodium hydroxide and the steam distillation resumed. The volatile, strong base fraction was collected in dilute hydrochloric acid. After concentration of the resulting solution to a syrup, the strong bases were liberated with potassium hydroxide and extracted with about 40 cc. of ether. The resulting extract was dried over KOH and then fractionated.

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[5] Fichter, F., and Labhardt, H. P., *Ber. chem. Ges.*, **42**, 4714 (1909).

The first fraction which consisted mainly of ether was condensed in a receiver chilled with ice and connected also with a dry ice trap. On addition of ethereal picric acid to the distillate and rinsings from the trap, copious crystallization occurred of the picrate which melted at 165–170°. The yield was 0.135 gm. and analysis showed it to be the salt of the base $C_7H_{15}N$.

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated, C 45.59, H 5.30; found, C 45.90, H 5.26

After transferral of the undistilled oil to a microstill, a rough fractionation was resumed and material was collected which distilled up to 145° at 760 mm. This fraction after dilution with ether yielded a picrate which was the salt essentially of the base $C_7H_{15}N$. After recrystallization from alcohol, 0.33 gm. of stout needles was obtained, which melted at 171–176°. 80 mg. of less pure picrate were recovered from the mother liquor. Finally, when the above distillation was carried further, it was found that the higher boiling material gave only inappreciable amounts of crystalline picrate. The total yield of crude picrate was roughly 0.57 gm.

After repeated recrystallization from alcohol a micromelting point of 176–178° was obtained.

$[\alpha]^{23}_{5780} = +9{,}7°$ ($c = 1.02$ in acetone)

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated. C 45.59, H 5.30, CH_3 4.39
Found. " 45.80, " 5.01, " 3.43

dl-N-Methyl-β-pipecoline was prepared according to Jacobi and Merling[3] by distillation of N-dimethyl-β-pipecolinium chloride. The crude base gave a picrate which after recrystallization from alcohol formed crystals which appeared broader than those from the cevine base and also had a lower melting point, *viz.* 165–168°. Found, C 45.95, H 5.20, CH_3 3.40.

However, since this synthetic material was racemic, an active β-pipecoline was first prepared according to Ladenburg[2] by cleavage with *d*-tartaric acid. *l*-Pipecoline-*d*-tartrate was thus obtained, which melted at 173–175°.

l-N-Dimethyl-β-pipecolinium iodide was prepared from *l*-β-pipecoline in methyl alcoholic solution with methyl iodide and sodium hydroxide. It formed prismatic needles from alcohol, which melted at 200–201° and showed $[\alpha]_D^{28} = +7.0°$ ($c = 1.0$ in water).

$C_8H_{18}NI$. Calculated, C 37.64, H 7.11; found, C 38.08, H 7.16

The chloride prepared from this salt with silver chloride was converted by distillation into *l*-N-methyl-β-pipecoline which was in turn isolated as the picrate. The latter after recrystallization from alcohol formed stout needles indistinguishable in appearance from the picrate obtained from cevine. It melted at 176–179°. However, $[\alpha]_{5780}^{23} = -12.6°$ ($c = 1.03$ in acetone). Found, C 45.77, H 5.44. Since the rotation of the picrate from cevine indicated that it contained a small amount of the racemic form, a mixture of 30 mg. of this picrate and 25 mg. of the above synthetic *l* derivative was recrystallized from alcohol. 47 mg. of a mixture were recovered, which appeared indistinguishable in form from the picrate of synthetic *dl*-N-methyl-β-pipecoline. Like the latter it melted at 164–168°, and, when mixed with it, showed no depression.

The free base recovered from the picrate from cevine was converted, for further characterization, into the quaternary salt with methyl iodide in ether solution. After recrystallization the resulting *d*-N-dimethyl-β-pipecolinium iodide melted at 199–201°.

$C_8H_{18}NI$. Calculated, C 37.64, H 7.11; found, C 37.70, H 6.72

At a subsequent stage of the work and following preliminary studies, further separation of the strong base fraction into secondary and tertiary bases was carried out over the nitroso compounds as follows.

Material was used which had been collected from a number of experiments, representing a total of 225 gm. of cevine which had been distilled with zinc dust. This included the mother liquors from the experiment described above, which gave 0.57 gm. of crude picrate of N-methyl-β-pipecoline. The mixture of strong bases in all of this material was again distilled with steam into dilute hydrochloric acid and the resulting solution was concentrated *in vacuo* to a syrup. This syrup of hydrochloride was dissolved in 10 cc. of water and treated with an excess of 30 per cent sodium nitrite solution. Oily nitroso compounds gradually separated. After standing at room temperature for 24 hours, the appreciable oily layer was extracted with a small volume of ether.

The aqueous fraction containing tertiary bases was made alkaline and distilled with steam into dilute hydrochloric acid. Concentration of the latter *in vacuo* left a partly crystalline residue of the hydrochloride. In a preliminary experiment a hydrochloride was obtained as needles from alcohol-ether, which melted at 180–192°. The analysis indicated it to be the salt of N-methyl-β-pipecoline.

$C_7H_{15}N \cdot HCl$. Calculated, C 56.15, H 10.78; found, C 55.44, H 10.70

However, because of its hygroscopic character the main material was converted into the picrate. After repeated recrystallization from alcohol, 0.95 gm. of picrate was obtained, which melted after preliminary softening at 165–171°.

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated, C 45.59, H 5.30; found, C 46.24, H 5.39

The ether solution of crude nitroso derivatives (described above) was mixed with an equal volume of alcohol and then an equal volume of concentrated hydrochloric acid. This mixture after standing at room temperature overnight was refluxed several hours and then concentrated to a syrup. This was desiccated by repeated concentration with absolute alcohol. The partly crystalline residue was finally dissolved in a small volume of absolute alcohol and on careful addition of dry ether yielded an oil followed by gradual crystallization which was increased by further addition of ether. The crystals were pipetted from persistent oily material and collected with ether. 0.85 gm. was obtained.

After recrystallization from alcohol-ether the salt formed needles which melted at 171°. No rotation could be detected in 1 per cent aqueous solution. Analysis showed it to be a pipecoline.

$C_6H_{13}N \cdot HCl$. Calculated. C 53.10, H 10.41, N 10.33
Found. " 53.17, " 10.46, " 10.30

For confirmation of its identity the base was converted into the 3,5-dinitrobenzoyl derivative by acylation in benzene solution with the acid chloride and dilute sodium hydroxide solution. The acyl derivative separated from 95 per cent alcohol as leaflets which melted at 111–113° after preliminary sintering.

$C_{13}H_{15}O_5N_3$. Calculated, C 53.22, H 5.16; found, C 53.17, H 5.04

This material showed no depression when mixed with 3,5-dinitrobenzoyl-β-pipecoline which melted at the same point and which was prepared from *dl*-β-pipecoline. The latter was obtained by hydrogenation of β-picoline which in turn had been prepared according to Stoehr[6] but finally purified as the picrate. The acyl compound gave the following analytical figures. Found, C 53.37, H 5.14. Attempts to identify any other base, and especially coniine, in the secondary base fraction have thus far been unsuccessful.

Soda Lime Distillation

In experiments involving distillation of 110 gm. of cevine with soda lime, as previously described, the fraction of bases boiling up to about 175° at 760 mm. as well as basic material recovered from the mother liquor of the picrate, $C_{10}H_{19}N \cdot C_6H_3O_7N_3$, were combined and finally obtained as a syrup of hydrochlorides. This material in concentrated solution was treated with 30 per cent sodium nitrite. Oily nitroso compounds gradually separated. After several hours the oil was extracted with a small volume of ether and hydrolyzed. After it was made alkaline, the secondary bases were steam-distilled into dilute hydrochloric acid and concentrated to dryness. In this case, dry ether added to the alcoholic solution precipitated first resinous material which was removed with pure bone-black. On further addition of ether to the filtrate and long standing, slow crystallization occurred. This was collected with ether. In view of the extremely small yield, about 50 mg., this material was acylated for characterization with 3,5-dinitrobenzoyl chloride. The reaction product was finally obtained as an oil which was treated with a drop or so of alcohol. All attempts to seed it with the β-pipecoline derivative were unsuccessful. However, the derivative of *d*-coniine caused prompt crystallization. After recrystallization from alcohol it slowly melted at 100–107° after preliminary sintering. It gave a strong depression with dinitrobenzoyl-β-pipecoline but not with dinitrobenzoyl-*d*-coniine.

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[6] Stoehr, *J. prakt. Chem.*, **43**, 154 (1890).

$[\alpha]_D^{25} = +11.7°$ (c = 0.515 in acetone)
$C_{15}H_{19}O_5N_3$. Calculated, C 56.04, H 5.96; found, C 55.87, H 5.62

The rotation of our previously described dinitrobenzoyl-*d*-coniine has since been found to be $[\alpha]_D^{25} = +49°$ (c = 1.025 in acetone).

In an attempt to confirm this result a similar fraction of bases resulting from the distillation of 70 gm. of cevine was similarly investigated.

In this case the concentrated solution of hydrochlorides of recovered bases was allowed to stand 24 hours after addition of sodium nitrite solution. The appreciable oily material which formed was extracted with ether and hydrolyzed. In this experiment a larger crystalline fraction was obtained than in the former case. 0.17 gm. of hydrochloride was collected from alcohol-ether and melted at 168–171° upon recrystallization.

$C_6H_{13}N \cdot HCl$. Calculated, C 53.10, H 10.41; found, C 52.94, H 10.48

The substance thus appeared to be the salt of β-pipecoline.

For further characterization the 3,5-dinitrobenzoyl derivative was made, which melted at 110–113°. It proved, however, to be optically active.

$[\alpha]_D^{30} = +8°$ (c = 0.5 in acetone)
$C_{13}H_{15}O_5N_3$. Calculated, C 53.22, H 5.16; found, C 53.20, H 5.06

In order to ascertain the optical character of this material the 3,5-dinitrobenzoyl derivative was prepared from *l*-β-pipecoline. It formed needles or leaflets which melted at 114–116° after preliminary sintering. $[\alpha]_D^{32} = -30°$ (c = 1.00 in acetone). Found, C 53.33, H 5.24.

Thus the rotation indicated that the above substance from cevine was the derivative of largely racemized *d*-β-pipecoline.

Further investigation of material contained in the mother liquors from this hydrochloride failed to give any certain indication of the presence of coniine. A small amount of crystalline material was obtained as a hydrochloride which proved to be an obvious mixture. Its analysis and the small amount of crystalline acyl derivative which could be obtained from it gave indications of a possible mixture of pipecoline and coniine or even of a dimethyl piperidine.

Oxidation of the Base $C_8H_{11}N$

0.2 gm. of the previously described picrate melting at 132°[1] was treated with 5 cc. of 10 per cent hydrochloric acid and picric acid was extracted with ether. The acid solution was evaporated to dryness *in vacuo* and the residue dissolved in a few drops of water. It was made neutral to litmus with potassium carbonate solution and treated with 0.6 gm. of potassium permanganate. The volume was then diluted to 30 cc. and the mixture was heated at 100° for 6 hours. The excess permanganate was removed with a slight excess of sodium bisulfite. The clear filtrate from manganese dioxide was exactly neutralized to Congo red with hydrochloric acid, then treated with 0.53 cc. of N hydrochloric acid, and finally evaporated to dryness. The residue was dissolved in 4 cc. of water. On chilling, 55 mg. of crystalline material slowly separated, which melted at 270–272° with vigorous decomposition when heated rapidly. Further recrystallization did not raise the melting point.

$C_7H_5O_4N$. Calculated, C 50.30, H 2.99; found, C 50.49, H 2.82

Decarboxylation of the Dibasic Acid—15 mg. of the acid were placed in a microstill[7] and heated under 25 mm. of pressure at an oil bath temperature of 270° until nothing more passed over. 11 mg. of crystalline material were removed mechanically from the condenser. This substance began to melt at 190–200°, but did not entirely melt until a temperature of 240° was reached. The distillate was again placed in the distillation apparatus and all material distilling up to an oil bath temperature of 150° and under 0.25 mm. of pressure was collected. It melted at 223–226°. The higher boiling material remaining in the residue gave more of the lower melting material upon being distilled again at the higher temperature as above. The material melting at 223–226° on redistillation at 150° and 0.25 mm. of pressure gave a product melting at 225–227°.

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[7] Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 219 (1936).

$C_6H_5O_2N$. Calculated, C 58.54, H 4.06; found, C 58.82, H 3.98

The three pyridine monocarbonic acids melt as follows: picolinic acid at 135°, nicotinic at 229–230°, and isonicotinic at 309°. Of the six possible pyridine dicarbonic acids, that substituted in the 3,5 positions melts at 322°,[8] and that in the 2,3 positions at 180° with decomposition.[9] The isomer substituted in the 2,4 positions could give only picolinic acid or isonicotinic acid upon decarboxylation, while that substituted in the 2,6 positions could give only picolinic acid. The acid from cevine described above must therefore be either the 2,5 or the 3,4 isomer. The 3,4 isomer gives upon decarboxylation a mixture of nicotinic and isonicotinic acids.[10] In the above decarboxylation experiment, however, no monocarboxy acid could be isolated which melted above 227°. Furthermore, the 3,4 derivative yields readily an anhydride when heated with acetic anhydride according to the directions of Strache.[11] Our acid gave only unchanged dibasic acid when subjected to this treatment. By a process of elimination the acid from cevine must therefore be the 2,5-pyridine dicarbonic acid or isocinchomeronic acid. Several values are given in the literature for its melting point, an uncertainty probably caused by its decomposition below the melting point to give nicotinic acid.[12]

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[8] Guthzeit, M., and Dressel, O., *Ann. Chem.*, **262**, 131 (1890).

[9] Hoogewerff, S., and van Dorp, W. A., *Ann. Chem.*, **204**, 117 (1880).

[10] Hoogewerff, S., and van Dorp, W. A., *Ann. Chem.*, **204**, 113 (1880).

[11] Strache, H., *Monatsh. Chem.*, **11**, 134 (1890).

[12] Weidel, H., and Herzig, J., *Monatsh. Chem.*, **1**, 16 (1879); **6**, 982 (1885).

THE SODIUM CONTENT OF BONE AND OTHER CALCIFIED MATERIAL*

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The sodium found in body tissues is now thought to be present in the extracellular fluid of the tissues, with little or no sodium within the cells (1). Analyses of various tissues by Harrison, Darrow, and Yannet (2) support this concept. However, in analyses of the skeleton a large quantity of sodium was found in excess of the amount accounted for by the extracellular fluid of the bone and cartilage. Part of this "excess sodium" is present in the cartilage, probably in combination with chondromucoid and can be extracted with water. Most of the excess sodium is found in the bone and cannot be dissolved by extraction with water or strong alkali solutions (2).

Since the studies of Gabriel (3) in 1894, the presence of significant amounts of sodium in bone has been noted but little attention has been paid to this finding. Logan (4) showed that Na plus K make up about 2.5 per cent of the bone base. The relatively large proportion of the body sodium found in the skeleton, *i.e.* 25 per cent of the total body sodium, stimulated our interest in the subject. The presence of sodium in bone has at times been explained as being due to adsorption of sodium bicarbonate or other salts on the precipitated calcium phosphate. However, the finding that the sodium of bone could not be eluted by boiling with water, alcohol, or 10 per cent potassium hydroxide solution suggested that it was not simply an adsorbate. The present experiments were designed to determine whether the sodium content of bone bore any relationship to the calcium content.

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In the first group of experiments the femurs of normal and rachitic rats, rats with osteoporosis, and rats given toxic doses of irradiated ergosterol were analyzed. Rickets was produced by feeding the Steenbock and Black diet (5). Animals given this diet with the calcium carbonate eliminated developed osteoporosis without the characteristic changes of rickets. All bone changes were confirmed by roentgenograms before the animals were sacrificed. Several groups of animals were fed 5000 to 10,000 international units of vitamin D daily for 3 weeks.[1] For purposes of analysis the animals were divided into groups of five. Before sacrificing the animals, blood was drawn anaerobically. The cleanly dissected femurs of each group of five were pooled for analysis. The blood serum was separated and the sodium, chloride, calcium, and phosphorus concentrations were determined by the Barber and Kolthoff (6), Van Slyke and Sendroy (6), Tisdall (7), and Benedict and Theis (6) methods respectively. The femurs were dried to constant weight in an oven at 105° and ground to a powder. Aliquot portions were taken for chloride determinations by the method of Van Slyke and Sendroy (6). The remainder was ashed at 500° in a muffle furnace and the calcium, phosphorus, and sodium determined in the dissolved ash by the McCrudden (6), Fiske and Subbarow (8), and Barber and Kolthoff (6) methods respectively.

The excess sodium of the bones is calculated as described by Harrison, Darrow, and Yannet (2). This may be briefly summarized in the following equations.

1. The volume of extracellular fluid of the bone equals the total chloride divided by the concentration of chloride in the extracellular water.
2. The sodium of the extracellular fluid equals the product of the volume of extracellular fluid and the concentration of sodium in extracellular water.
3. The excess sodium of the bone equals the total sodium minus the sodium of extracellular fluid.

The results of these analyses are graphically presented in Fig. 1 in which excess sodium is plotted against calcium, both expressed as mM per 100 gm. of fresh bone. Each point represents a group of five animals.

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[1] The concentrated irradiated ergosterol preparation was obtained through the courtesy of Dr. C. E. Bills of Mead Johnson and Company.

If the animals given large amounts of irradiated ergosterol be excluded temporarily from consideration, the results clearly show that the amount of so called excess sodium of bone is directly proportional to the amount of calcium in the bone. The molar ratio of calcium to sodium is approximately 30:1.

The bones of the animals given toxic doses of irradiated ergosterol show a quite marked deviation from the ratio, containing less sodium than expected.

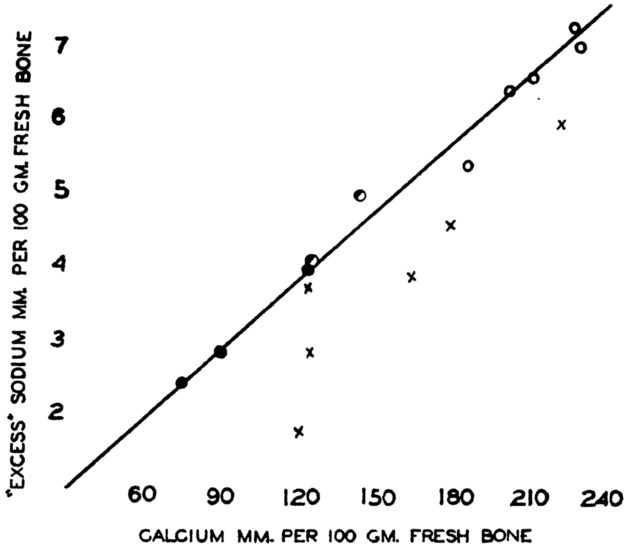

Fig. 1. Relationship of bone sodium to calcium. ○ normal, ◒ osteoporosis, ● rickets, × hypervitaminosis D.

To supplement the results cited above another type of experiment was performed. Bones of normal cats, a dog, and a monkey, tooth enamel from human teeth, a calcified cystic kidney, and a calcified tuberculous mesenteric node were analyzed in the following manner. The tooth enamel was washed repeatedly with distilled water and then dried. The various specimens of bone and calcified tissue were digested on the steam bath for 24 hours in a 5 per cent solution of KOH in 80 per cent alcohol. The residue was then washed repeatedly with hot alcohol and hot distilled water until the washings were no longer alkaline to phenolphthalein. The white, crystalline-looking residue was then dried.

An aliquot portion was analyzed for chloride. The remainder was dissolved in dilute HCl and determinations of Ca, P, and Na made. The methods used were identical with those previously mentioned. The results of these analyses are shown in Table I.

Except for the tooth enamel no chloride is present in the calcified material. The presence of appreciable quantities of chloride in tooth enamel agrees with other analyses (3, 9). All of the samples of bone or other calcified material contain sodium which cannot be dissolved by the procedures used to extract all organic material and water-soluble salts from the insoluble calcium salt. The insoluble sodium is present in fairly uniform concentration, the molar ratio of calcium to sodium being approximately 30:1 with a variation of about 10 per cent from the mean. The results of these analyses are in gratifying agreement with the indirect determination of excess sodium in the first group of experiments. This finding supports the theory involved in the calculation of the excess sodium and provides direct evidence that this fraction of the bone sodium represents a sodium salt different from that found in other tissues. It is also of considerable interest that tooth enamel and the two samples of pathological calcification contain insoluble sodium in approximately the same amounts as bone.

TABLE I

Analysis of Bone and Calcified Tissue

The substances are measured in mM per 100 gm. of insoluble residue.

Specimen	Ca	P	Cl	Na	Ca:Na
Cat femur	912	544	0	29.3	31.1
" "	908	538	0	28.5	31.9
Dog bone	608		0	18.7	32.5
Monkey bone	839		0	28.4	29.6
Human enamel	928	564	8.4	29.9	31.1
Calcified kidney	862	518	0	30.8	28.0
" lymph node	890	507	0	32.9	27.0

Any explanation for the presence of insoluble sodium in bone and calcified tissue requires knowledge of the nature of the calcium compound involved in calcification. Although dispute concerning the identification of this salt still exists, the investigations of Taylor and Sheard (10) and Roseberry, Hastings, and Morse (11) agree in their findings that bone has a crystalline structure which is similar to minerals of the apatite group. Bogert and Hastings (12) describe the bone salt as a carbonate apatite mineral with the formula $CaCO_3 \cdot nCa_3(PO_4)_2$ where the value of n is between 2 and 3. Other investigators (13) conceive of the bone salt as a mixture of hydroxyl and carbonate apatites.

Hansen (14) in a careful study of the naturally occurring apatite minerals noted that sodium in varying amounts was frequently found in samples of apatite. On the basis of quantitative analyses of fluorapatite he concluded that the sodium replaced some of the calcium in the molecule and that the composition was described by the following formula.

$$n3Ca_3(PO_4)_2 \cdot CaF_2$$
$$n3Ca_3(PO_4)_2 \cdot NaF$$

This expanation also accounts for the deficit of fluorine encountered in analyses of samples of fluorapatite. The alkali-containing apatite was found to have the same crystal structure as samples of fluorapatite free of sodium.

If Hansen's findings and conclusions are correct, the salt found in biological calcified material may be compared to sodium-containing fluorapatite. The constant ratio of calcium to sodium in the case of calcification may perhaps be explained by the fact that precipitation of the calcium salt takes place in a medium in which the concentration of sodium is quite constant. It is also theoretically possible that the sodium found in calcified tissue is not part of a complex salt but exists as a solid solution of a sodium salt in the apatite crystal. The similarity in the composition of samples of pathological calcification to that of bone indicates similarity in the conditions under which the various types of calcification occur despite the fact that quite different types of tissue are involved.

The deficiency in the amounts of sodium found in bones of animals given large doses of vitamin D is difficult of explanation. It may be connected with the decalcifying effects of toxic doses of vitamin D but this can only be a surmise at the present time.

SUMMARY

The finding has been confirmed that the sodium content of bone is greatly in excess of the amount accounted for by the extracellular fluid of the bone.

Analyses of the bones of rats with various disorders of calcification have demonstrated that with the exception of the bones of animals given large doses of irradiated ergosterol the "excess sodium" of bone is directly proportional to the calcium content, 1 molecule of sodium being found for 30 molecules of calcium. The bones of animals given toxic doses of vitamin D were found to contain less sodium.

The so called excess sodium of bones cannot be extracted by prolonged digestion with alcoholic potassium hydroxide solution and subsequent extraction with water.

Tooth enamel and two samples of pathologically calcified tissue were shown to contain approximately the same proportions of sodium to calcium as bone.

The suggestion is offered that the sodium found in calcified material is part of an apatite complex similar to the sodium found in the naturally occurring fluorapatite.

BIBLIOGRAPHY

1. Peters, J. P., Body water, Springfield (1935).
2. Harrison, H. E., Darrow, D. C., and Yannet, H., *J. Biol. Chem.*, **113**, 515 (1936).
3. Gabriel, S., *Z. physiol. Chem.*, **18**, 257 (1894).
4. Logan, M. A., *J. Biol. Chem.*, **110**, 375 (1935).
5. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).
6. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
7. Tisdall, F. F., *J. Biol. Chem.*, **56**, 439 (1923).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Bowes, J. H., and Murray, M. M., *Biochem. J.*, **29**, 221 (1935).
10. Taylor, N. W., and Sheard, C., *J. Biol. Chem.*, **81**, 479 (1929).
11. Roseberry, H. H., Hastings, A. B., and Morse, J. K., *J. Biol. Chem.*, **90**, 395 (1931).
12. Bogert, L. J., and Hastings, A. B., *J. Biol. Chem.*, **94**, 473 (1931-32).
13. Huggins, C., *Physiol. Rev.*, **17**, 119 (1937).
14. Hansen, H., *Acta Acad. Abœnsis*, **5**, 3 (1929).

SUGAR CONTENT OF HEPARINIZED AND OXALATED PLASMAS

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There are many studies indicating the influence of anticoagulants on the distribution of various ions in blood (1-3). Recently, Schmidt (4) has shown that, compared to heparinized plasma, oxalated plasma shows less phospholipid content. This is related to the changes in cell and plasma volumes produced by the oxalate. Sperry and Schoenheimer (5) have reached the same conclusion in a somewhat similar study on plasma cholesterol. Boyd (6) showed that the addition of potassium oxalate to defibrinated blood produced a shrinkage of red blood cells with an increase in their lipid content. Later he and Murray (7) made a thorough investigation of the effect of anticoagulants on the distribution of blood lipids. These last reports prompted the work on blood sugar herein reported.

Blood was obtained from healthy humans from a superficial arm vein; from rabbits, by cardiac puncture. After the sample of blood was drawn, it was divided into several parts by placing portions in heparin- and oxalate-coated centrifuge tubes to give the anticoagulant concentrations shown in Table I. Plasma values were obtained by Wintrobe's method (8). Sugar determinations were made by Benedict's method (9).

The results shown in Table I indicate clearly that, with human blood, changes in cell and plasma volumes due to the use of oxalates cause the free passage of sugar to or from the plasma accompanying the shift of the fluid. Potassium oxalate causes a shrinkage of the cell, produced by the removal of water from the cell. This shift of fluid from the cell, in the case of human blood, is accompanied by the passage of sugar from it. The

TABLE I

Effect of Anticoagulants on Plasma Sugar

Experiment No.	Plasma volume				Sugar per 100 cc.					Plasma sugar per 100 cc. whole blood			
					Whole blood	Plasma							
	Heparin, 0.014 per cent	$K_2C_2O_4$		$(NH_4)_2C_2O_4$, 0.3 per cent		Heparin, 0.014 per cent	$K_2C_2O_4$		$(NH_4)_2C_2O_4$, 0.3 per cent	Heparin, 0.014 per cent	$K_2C_2O_4$		$(NH_4)_2C_2O_4$, 0.3 per cent
		0.1 per cent	0.3 per cent				0.1 per cent	0.3 per cent			0.1 per cent	0.3 per cent	
						Human							
	per cent	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1		53.0	56.0	49.0	81		87	88	87		46	49	43
2	51.5	53.5			81	87	87			45	47		
3	51.5	53.5	55.5	48.0	81	86	86	85	86	44	46	47	41
4	52.0	54.0	57.0	49.0	78	83	83	82	82	43	45	47	40
5	51.0	53.5	56.0	48.5	92	100	99	99	98	51	53	55	48
6	49.0		54.5	46.5	77	82		81	81	40		44	38
7	55.0	57.0	59.0	52.0	89	95	94	95	95	52	54	56	49
8	53.0	54.5	58.0	49.5	86	93	92	92	93	49	50	53	46
9	50.5	53.0	56 0	47.5	82	89	88	88	88	45	47	49	42
10	49.0	51.5	53.5	46.0	61	65	65	65	65	32	33	35	30
11	50.0	52.5	55.5	47.5	70	78	78	77	78	39	41	43	37
12	51.5	53.5	56.0	47.5	91	98	98	98	98	50	52	55	47
13	52.0	54.5	57.5	49.0	72	77	78	77	77	40	43	44	38
14	50.5		55.5	48.0	80	85		85	84	43		47	40
15	50.5		56.0	47.5	75	80		79	80	40		44	38
16	49.0	52.0	53.5	46.0	81	88	89	90	89	43	46	48	41
17	47.0	50.0	52.5	44.5	77	83	83	83	82	39	42	44	37
						Rabbit							
1	65.0		69.0	62.5	114	152		145	158	99		100	99
2	66.0		69.0	62.5	97	127		122	133	84		84	83
3	62.0	63.0	65.0	57.5	94	131	128	124	139	81	81	81	80
4	65.0	66.5	68.0	61.0	95	127	125	120	133	83	83	82	81
5	69.5	70.5	71.5	66.0	114	147	144	144	154	102	102	103	102
6	63.0	64.5	66.0	59.5	94	123	120	114	129	77	77	75	77
7	58.5	60.0	61.5	54.0	130	184	182	175	196	108	109	108	106
8	68.0	69.0	70.5	65.5	114	145	144	141	150	99	99	99	98
9	63.5	64.0	66.0	59.0	94	121	120	118	127	77	77	78	75
10	68.5	69.5	70.5	65.0	127	158	156	155	164	108	108	109	107

reverse occurs with ammonium oxalate. This shows quite plainly that a free and ready permeability of human blood cells for sugar occurs.

On the other hand, our experiments indicate that rabbit blood cells differ markedly from human blood cells in their permeability for sugar. Depending upon the anticoagulant (potassium or ammonium oxalate), we find with rabbit blood (as with human blood) a dilution or concentration of plasma, but with no or very little shift of sugar.

The above facts regarding the permeability of rabbit and human blood cells for sugar are well known, but the method of study employed here is different from those usually used in such experiments and are therefore reported.

For aid in securing the samples of human blood, I am indebted to Dr. Brown; for the rabbit blood, to Mr. Reinhard. I am also grateful to those students from whom blood was obtained.

BIBLIOGRAPHY

1. Gaebler, O. H., *J. Biol. Chem.*, **99**, 99 (1932-33).
2. Blitstein, I., *Rev. belge sc. méd.*, **7**, 69 (1935).
3. Higounet, H., *Bull. Soc. chim. biol.*, **19**, 53 (1937).
4. Schmidt, L. H., *J. Biol. Chem.*, **109**, 449 (1935).
5. Sperry, W. M., and Schoenheimer, R., *J. Biol. Chem.*, **110**, 655 (1935).
6. Boyd, E. M., *J. Biol. Chem.*, **115**, 37 (1936).
7. Boyd, E. M., and Murray, R. B., *J. Biol. Chem.*, **117**, 629 (1937).
8. Wintrobe, M. M., *J. Lab. and Clin. Med.*, **15**, 287 (1929).
9. Benedict, S. R., *J. Biol. Chem.*, **92**, 141 (1931).

CHEMICAL STUDIES ON THE NEUROPROTEINS

II. THE EFFECT OF AGE ON THE AMINO ACID COMPOSITION OF HUMAN AND MAMMALIAN BRAIN PROTEINS

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(Received for publication, June 28, 1937)

It was pointed out in a recent publication (1) that the amino acid composition of the total protein fraction obtained from human, monkey, beef, sheep, rat, and guinea pig brains was quite constant. These experiments illustrated again the relative constancy of the amino acid, especially the basic amino acid, composition of the tissue proteins.

The experiments reported below extend and confirm the above conclusions. They describe the results of analyses of the proteins prepared from five normal human brains obtained from individuals varying in age from 4 to 82 years, as well as analyses of monkey, rat, and guinea pig brain proteins.

EXPERIMENTAL

Preparation of Human Brain Proteins—The fresh normal human brains were obtained as soon as possible from accident cases. In no instance was a brain used which had not been removed within 6 hours after death. Four of the five persons were accidentally killed by automobiles, while the fifth (aged 30 years) died from shock and submersion as the result of diving into cold water after severe overheating.

The proteins were prepared from the washed brains after removal of the meninges by either of the two procedures previously described (1). In confirmation of the earlier results, the proteins isolated by either method apparently have approximately the same amino acid composition.

Preparation of Animal Neuroproteins—These were prepared from pooled fresh or acetone-dried brains by the procedures described in Paper I of this series (1).

Analyses of Brain Proteins—The methods used for the determination of the amino acids were the same as described previously (1).

TABLE I

Amino Acid Composition of Mammalian Neuroproteins

Species	Sex	Age	Nitrogen	Histidine	Lysine	Arginine	Tyrosine	Tryptophane	Molecular ratio of lysine to arginine*
		yrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Human	Male	4	13.4	1.9	4.8	4.7	4.2	1.3	100:82
	"	14	15.1	2.7	5.3	5.1	4.5	1.4	100:81
	"	30 *Ca.*	15.1	2.5	5.2	5.4	4.6	1.4	100:86
	"	72	13.4	2.3	4.7	4.7	3.9	1.3	100:84
	"	82	13.0	2.5	5.0	5.3	4.1	1.4	100:89
Monkey	Unknown	Very young	12.8	2.1	4.8	5.4			100:95
	"	" "	14.7	1.9	5.2	5.4			100:87
Beef	Possibly female	Unknown	13.9	2.2	4.0	5.0			100:105
Sheep	" "	"	12.5	2.3	4.1	5.0			100:103
Rat	Unknown	Old	14.6	2.8	4.0	4.9			100:103
	"	"	14.3	2.7	5.5	5.7			100:87
	"	21-22 days	14.4	1.2					
Guinea pig	"	Unknown	13.8	1.9	5.1	5.6			100:92
	"	"	14.8	2.4	4.2	5.2			100:104

* These ratios are calculated from the analytical results carried to two decimal places.

Results

The proteins were prepared from five human brains of white males varying from 4 years to 82 years of age and dying of accidental causes. They were analyzed for nitrogen, histidine, lysine, arginine, tyrosine, and tryptophane. The analytical results together with some data published before (1) are summarized in Table I.

Inspection of the figures under "Molecular ratio of lysine to arginine" (Table I) indicates that *the lysine to arginine ratio is remarkably constant in the human neuroproteins throughout the entire age group.*

In contrast, it can be seen that the amount of histidine obtained from the brain protein prepared from the 4 year-old child is apparently less than that yielded by any of the other human protein preparations. The indication that there is less histidine yielded by acid hydrolysis of infantile brain protein preparations is supported by the finding that the amount of histidine obtained from 21 to 22 day-old rats is considerably less than that found in adult rat brains. The yield of histidine from the brains of young monkeys was also lower than that obtained from the other adults investigated. In this connection it is interesting that Okuda and Katai (2) reported that the cystine content of the hair of young individuals of six mammalian species was lower than that obtained from adults, while Smorodinzew and Bebeschin (3) say, "Die Altersveränderungen [of the human central nervous system] kommen am deutlichsten in den ersten 4–5 J. zur Äusserung."

DISCUSSION

Previous investigations have given the impression (*cf.* (4)) that the basic amino acids, especially arginine and lysine, must have some influence of particular significance in the genetic and embryological development of the tissue proteins. Thus it was found that the keratins, whether of vertebrate or invertebrate origin, yielded a relatively constant proportion of these amino acids (4). On the other hand, the entire protein fraction of blood serum, which has been called orosin (5), yields the same relative proportions of lysine to arginine in various mammalian species and this ratio is distinctly different from that observed among orosins of avian origin. The results reported in this paper indicate that the molecular ratio of lysine to arginine is constant in the neuroproteins prepared from the brains of normal human beings varying in age from 4 to 82 years. It appears from these and previous results, which are suggestive rather than conclusive, that the molecular ratio of lysine to arginine furnishes a tool for measuring similarities and differences in a series of homologous proteins.

SUMMARY

1. Proteins, prepared from the brains of five normal human males, varying in age from 4 to 82 years, were analyzed for nitrogen, histidine, lysine, arginine, tyrosine, and tryptophane.

2. The molecular ratio of lysine to arginine in the human neuroproteins remained remarkably constant throughout the entire age group.

3. Primate, rodent, and ungulate neuroproteins yield on hydrolysis approximately the same amounts of histidine, arginine, lysine, tyrosine, and tryptophane.

4. The data suggest that neuroproteins prepared from young mammals yield less histidine than those obtained from adults.

5. The importance of the molecular ratio of lysine to arginine and its possible bearing in comparative biochemistry is briefly discussed.

I am greatly indebted to Dr. A. Ferraro and Mr. F. Kramer of the Department of Neuropathology for furnishing me with the human material.

BIBLIOGRAPHY

1. Block, R. J., *J. Biol. Chem.*, **119**, 765 (1937).
2. Okuda, Y., and Katai, K., *J. Biochem.*, Japan, **24**, 207 (1936).
3. Smorodinzew, J. A., and Bebeschin, K. W., *J. Biochem.*, Japan, **24**, 245 (1936).
4. Block, R. J., *Yale J. Biol. and Med.*, **9**, 445 (1937).
5. Block, R. J., *J. Biol. Chem.*, **105**, 455 (1934).

DERIVATIVES OF *d*-GALACTURONIC ACID*

III. THE SYNTHESIS OF A MERCAPTAL OF *d*-GALACTURONIC ACID AND ALDEHYDO TETRAACETYLMETHYL-*d*-GALACTURONATE

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(Received for publication, June 11, 1937)

The existence of the aldehydo forms of the hexuronic acids has been postulated and inferred by analogy from the sugar group, but to date representative members have not been described (1–4). The researches of Levene and Meyer (5) and of Wolfrom (6) have shown that in the sugar group the mercaptals discovered by Emil Fischer (7) serve as excellent starting products for the synthesis of the aldehydo sugars.

Working along lines analogous to those employed by Emil Fischer with the sugars, we have been able to prepare the ethyl mercaptal of *d*-galacturonic acid (IV) and of its methyl ester (III). These are the first mercaptals realized so far in the hexuronic acid series. Acetylation of the mercaptal methyl ester led to the corresponding acetate (VI). Subsequent removal of the mercaptan residue through a graded hydrolysis comparable to that employed by Levene and Meyer (5) and later by Wolfrom (6) led to the isolation of the tetraacetylmethyl-*d*-galacturonate ethyl hemiacetal (IX). On repeated recrystallization from hot toluene, a molecule of ethyl alcohol is removed from the latter with the formation of the aldehydo tetraacetylmethyl-*d*-galacturonate (VIII).

The mercaptal of *d*-galacturonic acid (IV) shows one striking behavior that is worthy of note. On the basis of a postulation made in a previous paper of this series (8), it appeared that crystalline acylated derivatives of *d*-galacturonic acid or of a derivative with a free carboxyl group like α-methyl-*d*-galacturonide could not be realized unless the carboxyl was first protected by ester

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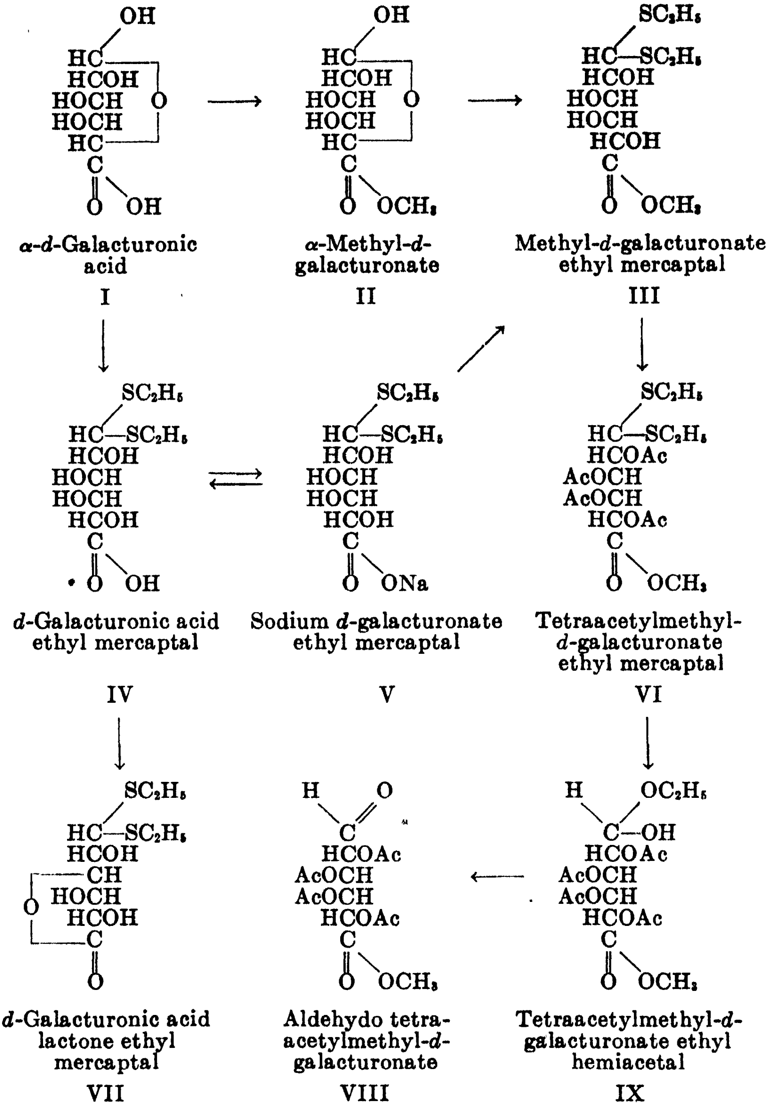
α-*d*-Galacturonic acid
I
α-Methyl-*d*-galacturonate
II
Methyl-*d*-galacturonate ethyl mercaptal
III
d-Galacturonic acid ethyl mercaptal
IV
Sodium *d*-galacturonate ethyl mercaptal
V
Tetraacetylmethyl-*d*-galacturonate ethyl mercaptal
VI
d-Galacturonic acid lactone ethyl mercaptal
VII
Aldehydo tetraacetylmethyl-*d*-galacturonate
VIII
Tetraacetylmethyl-*d*-galacturonate ethyl hemiacetal
IX

formation. The observations of Morell and Link (8) on *d*-galacturonic acid were independently confirmed by Dr. W. F. Goebel at the Hospital of the Rockefeller Institute working with *d*-glucuronic acid and its derivatives (p. 763). Contrary to the above, the mercaptal of *d*-galacturonic acid can be made readily from the free acid.

A lactone of the ethyl mercaptal (VII) was also obtained in a crystalline condition. This represents the first lactone derivative in the *d*-galacturonic acid series realized so far. Contrary to *d*-glucuronic acid which forms a lactone readily, all attempts to induce lactone formation with *d*-galacturonic acid or its derivatives had previously failed. Under conditions which lead to the glucuronolactone (heating in concentrated alcohols) *d*-galacturonic acid forms esters.[1] However, by heating (9, 10) a thin syrup of the ethyl mercaptal for 4 hours at 99° sufficient lactone formation was induced to permit its isolation in a pure condition. A dilute aqueous solution of the lactone maintained at 25° for 4 days showed no detectable hydrolysis. The optical rotation and the titer against 0.01 N sodium hydroxide remained constant.

The various transformations and syntheses described in this paper are given in the accompanying formulas.

An extension of this research to other hexuronic acids and a detailed study of the optical behavior of the free aldehydo derivatives of *d*-galacturonic acid is in progress.

EXPERIMENTAL

All analyses reported were made with the Pregl methods (11). The rotations reported were determined with a Franz Schmidt and Haensch polarimeter No. 52b with monochromatic light. The melting points were ascertained in a Thiele tube. The temperature was elevated at the rate of 2° per minute in the determinations.

Preparation of Sodium d-Galacturonate Ethyl Mercaptal

The method employed was essentially that of Emil Fischer (7) as modified by Levene and Meyer (5) and Wolfrom (6). 50 gm. of

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[1] When *d*-galacturonic acid ethyl mercaptal is recrystallized from hot water, products having a melting point range from 80–110° are obtained (mixtures of the free acid mercaptal and the lactone). By refluxing the acid mercaptal in anhydrous butyl alcohol, the butyl ester was obtained. The ester, recrystallized from an acetone-water mixture, showed a constant melting point of 104° and the correct composition for $C_{14}H_{28}O_6S_2$ (found C 47.8, H 8.0, S 17.7 per cent).

d-galacturonic acid were dissolved in 75 cc. of concentrated HCl by shaking in a glass-stoppered bottle. To the resulting amber solution 50 cc. of ethyl mercaptan were added and the mixture shaken. In a few minutes the temperature of the reaction mixture began to rise, whereupon it was cooled by immersion in ice water for approximately 5 minutes. Ice water (500 cc.) was then added. A light colored oil separated out, which was removed. The upper aqueous layer was then extracted by shaking with 800 cc. of ethyl ether. The ether layer was drawn off, combined with the original oil layer, and concentrated under reduced pressure to a thick syrup which was taken up in 100 cc. of 95 per cent ethanol. A concentrated solution of sodium hydroxide in methanol was then added until the mixture was alkaline to litmus. The crude sodium *d*-galacturonate ethyl mercaptal formed was collected by filtration and dissolved in 300 cc. of boiling ethanol to which enough water (approximately 130 cc.) was added to effect complete solution. After the alcoholic solution had cooled, the purified salt crystallized out in large hexagonal plates which were collected by filtration and dried over P_2O_5 under 12 mm. pressure. Yield 35 gm. Another fraction of the sodium *d*-galacturonate ethyl mercaptal was removed from the original HCl reaction mixture by addition of sodium hydroxide almost to neutrality and then an excess of sodium carbonate solution. The quantity of the recrystallized dried salt recovered at this stage was 14 gm., making an over-all yield of 49.0 gm. or 60 per cent of the theoretical.

Analysis—$C_{10}H_{19}O_6S_2Na$. Calculated, Na 7.14; found, Na 7.13
Rotation—$[\alpha]^{25}_{589.3} = -13.6°$ in water, $c = 5.24\%$

Conversion of Sodium d-Galacturonate Ethyl Mercaptal to the Free Acid

An excess of hydrochloric acid cooled to 0° was added to 35 gm. of the sodium *d*-galacturonate ethyl mercaptal. The liberated acid was collected by filtration, washed with cold water, and dried over P_2O_5 under 12 mm. pressure at 78°. Yield 26.5 gm. or 82 per cent of theory. The product contained traces of sodium which were removed by being dissolved in cold acetone (140 cc.). Cold water (35 cc.) was then added, after which the solution was rapidly concentrated under reduced pressure. Upon cooling, the acid crystallized out in fine needles. In a dilute water solution at room temperature (and much faster at elevated temperatures) the acid lactonized, which was indicated by a change in the rotation and by titer with 0.01 N alkali.

Analysis—$C_{10}H_{20}O_6S_2$

Calculated.	S 21.3, C 40.0, H 6.7
Found.	" 20.7, " 40.3, " 6.7

Melting Point—132.5°

Rotation—$[\alpha]^{25}_{589.3} = +17°$ in methanol, $c = 3.19\%$

Preparation of d-Galacturonic Acid Lactone Ethyl Mercaptal

An aqueous solution of *d*-galacturonic acid ethyl mercaptal (1 gm. in 30 cc. of water) was evaporated to a thin syrup (consistency of glycerol) by heating in an open dish on a boiling water bath. The heating extended over a period of 4 hours; then the syrup was placed in the ice chest for 5 hours, whereupon a crop of crystals separated which was a mixture of the free acid and the lactone. After filtration, the mother liquors were placed in the ice chest for 12 hours after which long transparent prisms (the pure lactone) separated. Owing to the high solubility of the lactone in the mother liquors, the product was collected as rapidly as possible by filtration (washed with the mother liquors) and dried over P_2O_5 at 25° under 12 mm. pressure. Yield 20 mg. (first crop).

Analysis—$C_{10}H_{18}O_5S_2$

Calculated.	C 42.6, H 6.4, S 22.7
Found.	" 43.1, " 6.6, " 22.8

Saponification Equivalent—No consumption of 0.01 N NaOH at 10°. 5.742 mg. consumed 2.10 cc. 0.01 N NaOH at 60–80°. Theory requires 2.04 cc.

Melting Point—79.0°

Rotation—$[\alpha]^{25}_{589.3} = +36°$ in water, $c = 0.83\%$

Preparation of Methyl-d-Galacturonate Ethyl Mercaptal

Method I—The substance was prepared from *d*-galacturonic acid ethyl mercaptal by direct esterification with diazomethane. To a solution of 7.5 gm. of *d*-galacturonic acid ethyl mercaptal in 100 cc. of absolute methanol, an ether solution of diazomethane was added until the reaction mixture was neutral to litmus. After a small flocculent precipitate was filtered off, the solution was concentrated under reduced pressure and cooled. The product which crystallized in the form of fine needles was collected by filtration, washed, and dried. Yield 4.0 gm.

Method II—The substance was prepared from *d*-galacturonic acid ethyl mercaptal by esterification with dry HCl-CH_3OH. To 50 cc. of a 0.25 per cent solution of dry HCl in absolute methanol were added 5.0 gm. of *d*-galacturonic acid ethyl mercaptal, and the solution refluxed for 40 minutes. The reaction mixture was concentrated (12 mm. pressure); the product taken up in acetone from which it crystallized directly (needles). Yield 2.0 gm.

Method III—The substance was prepared from methyl-*d*-galacturonate. Methyl-*d*-galacturonate (2.5 gm.) was dissolved in 4 cc. of concentrated HCl solution at 2° by shaking. Ethyl mercaptan (3 cc.) was added and the mixture shaken for about a minute, whereupon it set up as a solid cake. Ice water was then added to the crystalline product which was collected on a filter and purified by being recrystallized once from absolute methanol and two times from hot water. The yield of the purified product (fine needles) after drying over P_2O_5 under 12 mm. pressure was 1.5 gm.

Method IV—The substance was prepared from sodium *d*-galacturonate ethyl mercaptal by esterification with HCl-CH_3OH. To 150 cc. of dry 1.0 N HCl-CH_3OH mixture were added 30 gm. of sodium *d*-galacturonate ethyl mercaptal. The mixture was refluxed for 1 hour. The inorganic matter was removed (hot filtration) and the filtrate concentrated under reduced pressure, whereupon the product crystallized out (fine needles). Yield 21.5 gm. of the dried product.

The four different methods yielded products whose physical constants and analyses were in close agreement. A representative analysis follows.

Analysis—$C_{11}H_{22}O_6S_2$. Calculated, S 20.39; found, S 20.40

Saponification Equivalent—100 mg. consumed 32.00 cc. 0.01 N NaOH

Calculated Saponification Equivalent—100 mg. require 31.85 cc. 0.01 N NaOH

Melting Point—133-134°

Rotation—$[\alpha]^{25}_{589.3} = +17.8°$ in 95% ethanol, $c = 2.4\%$

Preparation of Tetraacetylmethyl-d-Galacturonate Ethyl Mercaptal

Methyl-*d*-galacturonate ethyl mercaptal (10 gm.) was dissolved in 35 cc. of dry pyridine. This solution was cooled in a water-ice bath, whereupon 50 cc. of acetic anhydride previously dried over sodium were added in small portions with shaking and cooling. After the temperature of the reaction mixture was held near 0° for 1 hour, room temperature (28°) was maintained for 18 hours. The reaction mixture was then poured into 2 liters of ice water, whereupon the product crystallized out. It was collected and dissolved in 70 cc. of hot methanol and the solution filtered hot; upon cooling, 12 gm. of the pure substance crystallized out. An additional 1 gm. was obtained after addition of water to the mother liquor.

Analysis—$C_{19}H_{30}O_{10}S_2$. Calculated, S 13.3; found, S 13.2
Saponification Equivalent—100 mg. consumed 10.60 cc. 0.1 N NaOH
Calculated Saponification Equivalent—100 mg. require 10.40 cc. 0.1 N NaOH
Melting Point—112.5–113.5°
Rotation—$[\alpha]^{25}_{589.3} = +20.5°$ in U.S.P. chloroform, $c = 5.5\%$

Preparation of Aldehydo Tetraacetylmethyl-d-Galacturonate Ethyl Hemiacetal

To a mixture of 30 cc. of acetone and 4 cc. of water in a 3-necked, round bottom flask equipped with a reflux condenser and a stirrer, were added 8.3 gm. of tetraacetylmethyl-*d*-galacturonate ethyl mercaptal. After complete solution was attained, 15 gm. of washed cadmium carbonate and a solution of 16 gm. of mercuric chloride in 24 cc. of acetone were added. The mixture was agitated for 24 hours at room temperature (28°), whereupon it was increased to 50° and held for 15 minutes. The mixture was then brought to the boiling point for 15 minutes and filtered hot. The filtrate was concentrated to dryness under reduced pressure in the presence of cadmium carbonate. This concentrate was dried by being taken up repeatedly (three times) with absolute ethanol. The residue from the alcohol concentrate was extracted with warm chloroform, and to the chloroform solution 20 cc. of absolute ethanol were added. After removal of the alcohol-chloroform mixture under reduced pressure, the aldehydo acetylmethyl-*d*-galacturonate ethyl hemiacetal crystallized out. It was collected, washed with absolute ethanol, and dried over P_2O_5 under 12 mm. pressure. Yield 3.8 gm.

Analysis—$C_{17}H_{26}O_{12}$

Calculated, alkoxyl 18.00; found, alkoxyl 17.97

Saponification Equivalent—100 mg. consumed 12.46 cc. 0.1 N NaOH

Calculated Saponification Equivalent—100 mg. require 11.90 cc. 0.1 N NaOH

Melting Point—Sintering at 113°, melts at 139°

Rotation—$[\alpha]^{25}_{589.3} = +16.5°$ after 10 minutes; $-3.0°$ after 36 hours in tetrachloroethane, $c = 3.9\%$

Preparation of Tetraacetylmethyl-d-Galacturonate Semicarbazone

The aldehydo tetraacetyl ethyl hemiacetal (0.84 gm.) was dissolved in 15 cc. of hot water. The solution was cooled to 28° and a mixture of 0.20 gm. of semicarbazide hydrochloride and 0.32 gm. of potassium acetate was added. After solution of the mixture was effected by shaking, it was placed in the ice chest, whereupon the crystalline semicarbazone separated. The semicarbazone was recrystallized three times from hot water to which sufficient ethanol was added to effect complete solution.

Analysis—$C_{16}H_{23}O_{11}N_3$. Calculated, N 9.73; found, N 9.71

Saponification Equivalent—100 mg. consumed 11.68 cc. 0.1 N NaOH

Calculated Saponification Equivalent—100 mg. require 11.55 cc. 0.1 N NaOH

Melting Point—219–220° (decomposition)

Rotation—$[\alpha]^{25}_{589.3} = +83.4°$ in chloroform, $c = 4.0\%$

Aldehydo Tetraacetylmethyl-d-Galacturonate from the Hemiacetal

After 0.5 gm. of the tetraacetylmethyl-*d*-galacturonate was recrystallized three times from hot toluene, the free aldehydo compound was obtained as crystalline, colorless needles.

Analysis—$C_{15}H_{20}O_{11}$. Calculated,—OCH_3 8.25; found,—OCH_3 8.25

Melting Point—136.5–137.5°

Rotation—$[\alpha]^{25}_{589.3} = -16.2°$ in tetrachloroethane, $c = 2.2\%$

SUMMARY

1. The preparation of the ethyl mercaptal of *d*-galacturonic acid, its lactone, and the methyl ester of *d*-galacturonic acid ethyl mercaptal are described. They represent the first mercaptals in the hexuronic acid series; the lactone, the first lactone of the *d*-galacturonic acid series.

2. The ethyl mercaptal of *d*-galacturonic acid methyl ester acetylated readily to form the corresponding acetyl derivative. Removal of the mercaptan residue through a graded hydrolysis resulted in the formation of the ethyl hemiacetal of 2, 3, 4, 5-tetraacetyl-*d*-galacturonic acid methyl ester.

3. The free aldehydo 2, 3, 4, 5-tetraacetyl-*d*-galacturonic acid methyl ester was prepared from the ethyl hemiacetal.

4. The compounds were all obtained in a crystalline condition and the physical constants and analysis are reported.

BIBLIOGRAPHY

1. Abderhalden, E., Biochemisches Handlexikon, Berlin, **13**, suppl. 6 (1931).
2. Vogel, H., and Georg, A., Tabellen der Zucker und ihrer Derivate, Berlin (1931).
3. Tollens, B., and Elsner, H., Kurzes Handbuch der Kohlenhydrate, Leipsic, 4th edition (1935).
4. Ehrlich, F., and Schubert, F., *Ber. chem. Ges.*, **62**, 1974 (1929).
5. Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **69**, 175 (1926); **74**, 695 (1927).
6. Wolfrom, M. L., *J. Am. Chem. Soc.*, **51**, 2188 (1929); **52**, 2467 (1930); **54**, 3390 (1932) and subsequent papers in same journal.
7. Fischer, E., *Ber. chem. Ges.*, **27**, 673 (1894).
8. Morell, S., and Link, K. P., *J. Biol. Chem.*, **108**, 763 (1935).
9. Schoeffel, E., and Link, K. P., *J. Biol. Chem.*, **100**, 397 (1933).
10. Hedenburg, O. F., *J. Am. Chem. Soc.*, **37**, 345 (1915).
11. Pregl, F., revised by Roth, H., Die quantitative organische Mikroanalyse, Berlin, 4th edition (1935).

THE ESTIMATION OF ALBUMIN AND GLOBULIN IN BLOOD SERUM

I. A STUDY OF THE ERRORS INVOLVED IN THE FILTRATION PROCEDURE*

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(Received for publication, June 3, 1937)

In the determination of the "albumin" and "globulin" fractions of blood serum by precipitation with empirical concentrations of various salts, too little attention has been paid to the details of filtration, even though there is general agreement (1, 2) that removal of the precipitated globulin is the step most difficult to control. In many reports there is no mention of the type of filter paper or the number of times the albumin filtrate was refiltered. With the Howe micromethod (3) (precipitation of globulin in 1.50 M, 21.3 per cent, solution of sodium sulfate at 37°), we noted that often when it was necessary to refilter one albumin filtrate more times than its duplicate the latter always contained a higher concentration of albumin. This led us to study the influence on the protein concentration in the filtrate of, first, the type and amount of filter paper; secondly, refiltration through the same paper; and thirdly, the quantity of the serum-sodium sulfate mixture that is filtered through the same paper. The results prove that filter paper, in the presence of high salt concentration, does adsorb an appreciable amount of albumin and, therefore, the values for albumin are low, especially when small amounts of solution are filtered. This error in albumin determination is magnified when albumin to globulin ratios are calculated.

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* An abstract of this paper was presented before the Thirty-first annual meeting of the American Society of Biological Chemists at Memphis, April, 1937.

EXPERIMENTAL

Human, dog, and rabbit sera were used in these experiments. Dog and rabbit blood from apparently normal animals was obtained from the heart without anesthesia and human blood from the veins of hospital patients. After clotting and centrifuging, the blood serum was removed at once from the cells and treated with the 22 per cent solution of sodium sulfate within 3 hours after the blood was drawn, after which the mixture was allowed to stand at 37° overnight. Protein appears to be stable in this strong salt solution, for analysis of the nitrogen content of the filtrate filtered 4 hours after the mixing agrees with that filtered after standing for 48 hours.

The types of filter papers recommended for this filtration and tested in this investigation include Whatman No. 5, No. 50, and No. 42, Schleicher and Schüll No. 575, Munktell No. 00, and Delta No. 325. Usually the albumin filtrate must be refiltered several times through the same filter paper before it is clear. The number of refiltrations was markedly reduced when two sheets of the paper were folded together in the funnel instead of a single sheet, but, as will be shown, this caused an increased error.

In the Howe micromethod 15 cc. of the 22 per cent sodium sulfate solution are added to 0.5 cc. of serum and from the filtrate duplicate nitrogen determinations are made on 5 cc. portions. Throughout this work, except in the experiment reported in Table I, a filter paper 9 cm. in diameter was used on a 55 mm., 60° angle, Pyrex fluted funnel. This filter holds the entire 15.5 cc. sample. When larger amounts of the mixture were filtered through the same filter, 15 cc. samples were placed on the filter at one time and allowed to filter completely. Since a clear filtrate is generally not obtained until the pores of the filter paper are filled with the precipitated globulin, it is always advantageous to shake up the precipitate in the serum-salt mixture before it is poured on the filter. Total serum protein and non-protein nitrogen determinations were made on all samples, so that the changes in the albumin to globulin ratio might be observed.

Methods

Total Serum Protein—0.5 cc. of serum was measured, with a Van Slyke-Ostwald "between marks" pipette, into a 25 cc. volumetric flask and made up to volume with a 0.9 per cent solution of sodium chloride. When the serum was plentiful, larger amounts were diluted. Four 5 cc. aliquots were measured into 100 cc. Pyrex Kjeldahl flasks to which were added 3 cc. of a sulfuric acid-selenium oxychloride digestion mixture (1 cc. of selenium oxychloride added to a mixture of 250 cc. of concentrated sulfuric acid and 250 cc. of a saturated solution of potassium sulfate) and two small glass beads. The flasks held in the digestion rack at an angle of 45° were heated gently until the water was removed and the contents were clear, and then strongly for 20 minutes. The distillation of the ammonia into 0.01 N HCl solution was carried out in the Goebel modification of Pregl's micro-Kjeldahl distillation apparatus (Peters and Van Slyke (1) p. 531). Three stills were run simultaneously. It takes from 7 to 10 minutes to carry out the distillation, and, while a set is being distilled, the excess acid in the previous group can be titrated with approximately 0.01 N alkali, in the presence of methyl red as the indicator. Nitrogen determinations on the reagents were made at frequent intervals. The protein concentrations were calculated from the difference between the total nitrogen and the non-protein nitrogen values by multiplying by 6.25.

Non-Protein Nitrogen—The protein was precipitated by trichloroacetic acid. Usually 2.5 cc. of serum were introduced into a 25 cc. volumetric flask containing about 20 cc. of a 10 per cent solution of trichloroacetic acid. The flask was rotated continuously as the serum was added slowly, then the mixture was made up to volume with the acid solution. The protein precipitate was removed by centrifugation followed by filtration. Two 10 cc. samples were placed in Kjeldahl flasks and treated as described under "Total serum protein."

Albumin—A solution of 22 per cent sodium sulfate was added to the serum at 37° in the proportion of 30 parts of the salt solution to 1 part of serum. It was mixed thoroughly and allowed to stand overnight in a 37° incubation room in which all apparatus was kept as recommended by Bruchman, D'Esopo, and Peters (4). At 37° the film that remains on the wall of the pipette dries rapidly and salt and protein may separate out so that if another sample is measured with this pipette, this residue may be taken up and the state of equilibrium of the solution disturbed. In order to avoid this condition, a clean 5 cc. pipette was used for each 15 cc. portion of filtrate, and measurements were made as quickly as possible. 5 cc. aliquots of the filtrate were used for the nitrogen determinations which were carried out as already described. 10 minutes of vigorous boiling after the contents of the flask have become clear is sufficient for complete digestion; moreover, longer boiling may cause a loss of acid and precipitation of salt which results in dangerous bumping. It is worth noting that more than 5 cc. aliquots of the filtrate are not satisfactory with a 100 cc. Kjeldahl flask. All the filter papers used contain a nitrogen compound which is practically all removed by the first 15 cc. of solution. Therefore, when aliquots of a filtrate from the first 15 cc. are analyzed, a nitrogen determination should be made for each lot of paper. This correction is negligible in most cases and not necessary in the modification of the method to be proposed. The concentration of globulin is calculated by the difference between the total serum protein and the albumin values.

With the above methods our determinations of total protein and albumin have a precision within ±0.03 gm. per 100 cc.

Results

Influence of Quantity and Type of Filter Paper—The experiment summarized in Table I is representative of many illustrating the influence of size and number of sheets of filter paper on the removal of albumin. In this particular case a clear filtrate was obtained without refiltering and it is certain that the high value in the filtrate which passed through only one thickness of paper was not due to turbidity. There was a progressive loss of protein in the filtrate with increase of the number of sheets of filter paper, but less loss in protein when the diameter of the filter paper is reduced. The amount of albumin adsorbed is a function of the amount of paper that comes in contact with the solution.

It is clear from these results that the use of two thicknesses of paper with the usual technique is to be condemned. Also, a refiltration through a fresh paper should never be made, as the new paper will adsorb an additional amount of the albumin. This effect is illustrated in the fourth column of Table I. When the filtrate was passed through a new filter of two sheets of the paper, after it had already been through such a filter, the albumin dropped from 2.54 gm. per cent to 2.23 gm. per cent.

TABLE I

Influence of Quantity of Filter Paper on Albumin Value When 15.5 Cc. of Serum-Sodium Sulfate Mixture Are Filtered Through Whatman No. 5 Paper

Dog serum was used. Albumin values are expressed in gm. of protein per 100 cc. of serum.

	9 cm. diameter				5 cm. diameter
	1 sheet	2 sheets	3 sheets	2 sheets + 2 sheets	2 sheets
Albumin	2.95	2.54	2.34	2.23	2.82
" to globulin ratio	1.05	0.79	0.68	0.63	0.96

TABLE II

*Effect of Filtering Through Various Types of Filter Paper**

The quantity of solution filtered in each case is 15.5 cc. All filter papers are 9 cm. in diameter.

Serum	No. of sheets and type of filter paper	No. of times filtered	Serum albumin	Albumin to globulin ratio
			gm. per 100 cc.	
Human	2, No. 5	3	4.20	1.27
	1, " 00	3	4.83	1.80
	1, " 50	3	4.92	1.89
	1, " 575	3	4.95	1.93
Rabbit	1, " 50	2	4.51	2.21
	1, " 5	2	4.42	2.08
	1, " 42	2	4.43	2.09
	2, " 50	1	4.37	2.00
	2, " 5	1	4.20	1.79
	2, " 42	1	4.30	1.91

* The filter paper Nos. employed throughout this paper are those used by the manufacturers: Munktell No. 00, Whatman No. 5, 50, and 42, Schleicher and Schüll No. 575, Delta No. 325.

During the search for a filter paper that would give a clear filtrate without the necessity of any refiltration, a number of types of paper were studied. Two representative experiments are shown in Table II. All papers take out albumin, although there is a difference in the amounts that are adsorbed by the different types; the so called hardened papers, such as Whatman No. 50 and Schleicher and Schüll No. 575, take out less than the soft papers. Table II also illustrates the marked differences in the albumin to globulin ratio that may be obtained by changing the type and quantity of the filter paper.

Effect of Refiltration Through the Same Paper—It has been previously stated that with most blood sera the albumin filtrate must be refiltered several times before a clear filtrate is obtained. Since it was noticed in our records that refiltration might be a factor in producing lowered values, experiments were carried out to test more directly the effect of refiltration. As most samples do not become clear with the first filtration, determinations on filtrates which were not perfectly clear would give results influenced by the two opposing errors, the adsorption of protein by the filter paper and traces of precipitated globulin in the filtrate. Representative experiments are recorded in Table III.

TABLE III

Effect of Refiltration

Albumin concentration in serum from determinations made on filtrates from 15.5 cc. samples of serum-sodium sulfate mixtures when these samples were refiltered a number of times after becoming clear. The concentrations are in gm. per 100 cc. of serum.

Serum No.	No. of sheets and type of paper	Clear filtrate value	No. of times refiltered after becoming clear				
			1	2	3	4	5
			gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
1 (Dog 3)	2, No. 5	3.25(1)*	3.13	3.04	2.94	2.78	2.77
2 (" 1)	1, " 50	4.36(x)	4.30	4.26	4.26	4.24	4.24
	2, " 5	3.72(x)	3.53	3.49	3.40	3.34	3.36
3 (Human 21)	2, " 5	0.90(2)	0.87	0.79	0.76	0.69	0.68
4 (Dog 1) clear filtrate	2, " 5	3.50(1)	3.36	3.30	3.24	3.21	3.19

* Figures in parentheses indicate the number of times that samples were refiltered before a clear filtrate was obtained; (x) indicates a portion was poured back on the filter before the entire sample had been filtered, therefore it is uncertain how many times these samples were refiltered.

Large amounts of the serum-sodium sulfate mixtures were prepared and after thorough shaking aliquot 15 cc. portions were placed on each of a series of filters set up as represented by the successive columns in Table III. The entire series of samples was then treated in exactly the same manner until all the filtrates were clear. Then duplicate aliquots from the first filter were analyzed to give the value recorded in the column marked "Clear filtrate value." On the next filter the filtrate was refiltered once, on the next one refiltered twice, and so on as shown in Table III. In the first serum (Dog 3) a clear filtrate was obtained with the first filtration through two sheets of paper. In each of the next four refiltrations protein was removed after which no more protein was adsorbed by the paper. With Serum 2 the samples had to be refiltered a number of times before a clear filtrate was obtained. Portions of the filtrate were placed back on the filter before the entire sample had come through, so that there can be no definite figure given for the number of times the sample was refiltered before becoming clear. This probably explains why equilibrium is reached here much sooner than in Serum 1. Serum 3, from a nephrosis patient, is an example of a serum with an extremely low albumin concentration.

Serum 4 (Table III) was handled differently from the others. A large amount of the serum-sodium sulfate mixture was filtered until clear on a number of filters. The filtrates were pooled, mixed thoroughly, and 15 cc. samples were put through a set of filters corresponding to the other experiments in Table III. The first sample was filtered once, the next sample two times through the second filter, and so forth until a fifth 15 cc. sample was filtered five times through the fifth filter. This experiment is important because it illustrates the fact that the filter paper takes out albumin in the *absence* of globulin precipitate on the filter.

These experiments, which are illustrative of many other determinations, show definitely that refiltration takes out an increasing amount of protein up to a limit, after which no more protein is adsorbed by the paper. The number of refiltrations that are necessary to satisfy this property of the paper apparently depends upon the length of time of contact between solution and paper, which in turn depends upon rate of filtration.

Effect of Quantity of Solution—It was found that successive portions of the filtrate from the 15.5 cc. serum-sodium sulfate mixture were not uniform in protein concentration. The first 5 cc. contained much less protein than the next 5 cc. From this finding, and from the fact that the amount of protein taken out by the paper was limited (Table III), it seemed evident that filtering larger quantities of solution through the same filter paper should raise the albumin concentration in the filtrate. That this is true is evident from a typical experiment shown in Table IV. The protein concentration of the filtrate is dependent on the amount of solution that is filtered. In this experiment the albumin to globulin ratio was changed from 1.06 to 1.38 by allowing an additional 31 cc. to pass through the paper.

TABLE IV

Effect of Increasing Quantity of Solution Filtered

Serum of Dog B-3 was used throughout.

Two sheets of Whatman No. 5 paper, 9 cm. in diameter, were used in each determination. Aliquots for analyses were taken from the entire filtrate of the quantities of mixture indicated.

Quantity of mixture filtered	Serum albumin	Albumin to globulin ratio
cc.	*gm. per 100 cc.*	
15.5	3.02	1.06
31.0	3.32	1.31
46.5	3.40	1.38

Instead of analyzing the total filtrate in our next experiments, we made determinations on successive 15 cc. portions. The results are summarized in Table V. The albumin concentration was found to increase in each fraction until 30 to 45 cc. had been filtered. When additional 15 cc. portions were filtered, the albumin value remained *constant*. Apparently, after 30 to 45 cc. of solution have been in contact with the filter paper, the paper becomes saturated with albumin and, therefore, successive filtrates represent the actual concentration of albumin in solution at this concentration of salt. This value will be referred to in the rest of this paper as the "correct" value. The experiments presented below in Table VII also strengthen this conclusion that the filter paper becomes saturated with a definite amount

TABLE V

Albumin Concentration of Blood Serum, Calculated from Analyses of Successive 15 Cc. or 30 Cc. Portions of Filtrate,

The number of filtrations necessary to obtain a clear filtrate is indicated by the figure in parentheses. All filter papers were 9 cm. in diameter. The results are expressed as gm. per 100 cc. in original serum.

Serum	No. of sheets and type of filter paper	Analyses of successive 15 cc. portions of filtrate*						
		1st	2nd	3rd	4th	5th	6th	7th
Human								
1	1, No. 00	4.83(3)	5.07(2)	5.10	5.09	5.10	5.11	
	1, “ 50	4.92(3)	5.03(2)	5.08(2)	5.10	5.10	5.09	
	1, “ 575	4.95(3)	5.00	5.05	5.09	5.09		
2	1, “ 00	2.55(2)	2.68(2)	2.73	2.72	2.73	2.71	
14	1, “ 50	3.77(x)†	3.90	3.86	3.90	3.92	3.92	
	2, “ 5	3.24(x)	3.70	3.80	3.83	3.84	3.93	3.92
8	1, “ 50		4.16(2)‡		4.24‡		4.23‡	
20	1, “ 50		2.25(5)‡		2.37(3)‡		2.38(3)‡	
	2, “ 50		2.15(5)‡		2.34(3)‡		2.35(3)‡	
Dog								
1-1	1, No. 00	3.71(2)	3.94	3.94	3.92	3.93	3.94	
	1, “ 50	3.76(2)	3.85	3.87	3.88	3.94		
	1, “ 575	3.79(2)	3.95	3.86	3.89	3.92		
1-2	1, “ 50		3.82(x)‡		3.91‡		3.88‡	3.88
3-2	1, “ 50		4.26(3)‡		4.38(2)‡		4.37(2)‡	
Rabbit								
1	1, No. 00	4.35(2)	4.53	4.55	4.55	4.58	4.57	
	1, “ 50	4.39(3)	4.55	4.55	4.58	4.55	4.57	
	1, “ 575	4.42(2)	4.48	4.57	4.56	4.58	4.57	
3	1, “ 50		4.68(x)‡		4.85‡		4.83‡	
	1, “ 5		4.56(x)‡		4.78‡		4.81‡	

* Each value represents the average of at least two, and usually four determinations from aliquots of the same filtrate or filtrates from duplicate precipitations. The standard deviations, calculated by the formula S.D. = $\sqrt{\Sigma d^2/(n-1)}$, averaged ±0.03 and were never greater than ±0.05.

† (x) indicates the number is not known because a portion was poured back on the filter.

‡ Values are for 30 cc. portions.

of protein. In a comparative study of different papers the values of the albumin were, in some cases, more than 0.6 gm. per 100 cc. apart in the first 15 cc. portions but reached the same value in later portions on all papers.

Further evidence of the significance of the "constant" albumin value was given in what we call our "Flood" experiment. When work in the laboratory had to be curtailed because of failure of the water supply during the Ohio Valley flood, 465 cc. of a serum-salt mixture were ready for filtration. This stood in a liter flask in the 37° room for over 2 weeks, by which time the supernatant liquid was perfectly clear. A 25 cc. sample of the supernatant liquid was withdrawn with great care to avoid disturbance of the precipitate. The albumin content of the supernatant liquid agreed perfectly with samples filtered by the technique given above.

The influence of the amount of solution filtered may also explain the conclusions of Howe (5) in regard to loss of protein by adsorption, "In using the procedures described it is assumed that the aliquot taken from the filtrate after precipitation contains a true proportionate amount of the unprecipitated protein and that there has not been any adsorption of the unprecipitated protein by the precipitated protein nor by the filter paper." In his determinations of the critical zone for the precipitation of total globulins, 5 cc. portions of blood plasma were added to 50 cc. portions of sodium sulfate solution. Thus, about 55 cc. of solution were filtered and the effects of the filter paper may have been small.

In Table V are given a few representative experiments. Human 1, Dog 1, and Rabbit 1 serve as parallel tests with the three most suitable filter papers. It is important that, even though the filtrate had come through perfectly clear after two to four refiltrations of the first sample, the filtrates from the next portions often showed a turbidity that necessitated refiltration, but this refiltration certainly does not remove albumin; in contrast to refiltration before the paper has adsorbed its maximum amount.

These data also show the relationship between the error involved in filtering 30 cc. portions as compared with 15 cc. portions. It is quite evident that the error is less when 30 cc. portions are filtered. When one sheet of Whatman No. 50 paper is used with

30 cc. portions, the value of the albumin determination on the serum is still about 0.1 gm. per 100 cc. below the "correct" value. In the discussion of Table I, where it was shown that two sheets of filter paper took out more albumin than one, the use of more than one sheet was condemned. However, the results show that the same process of successive filtrations may be employed with two sheets, for the same "correct" value is reached irrespective of the number of sheets used, but it does generally take longer to reach the equilibrium.

Albumin Determinations on Diluted Serum—Howe (5) noticed when his micromethod was used with diluted samples of serum that a proportionate amount was never recovered. He stated, "when diluted serum is filtered...there is a small loss of nitrogen in the process which may be slightly greater than the experimental error." This observation has been confirmed and extended by Goettsch and Kendall (6) who concluded, "In each case precipitation of the globulin in the diluted serum was more complete than in the original serum and resulted in low values for albumin." We have also noted the same phenomenon with diluted serum, as shown in the results given in Table VI. Incidentally, this work was done before we had decided against the use of two sheets of paper. However, the results are not only convincing but have the advantage of being a more severe test of the adsorption of albumin than would be the case with one sheet. It is evident from Columns 5 and 6 that with greater dilutions of serum a greater proportion of albumin is lost, as is indicated by the great decrease in the albumin to globulin ratio. In the case of the concentrated rabbit serum the ratio dropped from 2.26 to 1.61 when the serum was diluted 1 : 5.

Column 7 of Table VI gives the "correct" values that were determined on the undiluted samples with large amounts of sera, as described in the preceding experiments (Table V). Column 8 gives the values for albumin calculated on the basis of the dilution from this "correct" value. When the determined albumin values in Column 5 are subtracted from the calculated values of Column 8, one obtains the amounts of albumin, in terms of gm. per 100 cc. of sample, which were taken up by the filter paper. It is evident that the values for each serum at different dilutions agree well within the limit of error of this technique. Therefore, it must

TABLE VI

Effect of Serum Dilution on Albumin Determination

Sera were diluted in the proportion indicated with a 0.9 per cent sodium chloride solution. To the serum and diluted samples a 22 per cent solution of sodium sulfate was added in the proportion of 1:30. The rabbit serum was concentrated by ultrafiltration (7) before the dilution with saline. A portion of the dog serum was concentrated by the same procedure.

Serum (1)	Size of sample, No. of sheets, and type of paper (2)	Dilution serum-saline (3)	Determined total protein (4)	Determined albumin (5)	Albumin to globulin ratio (6)	Determined albumin "correct" value (7)	Albumin calculated from "correct" value (8)	Albumin adsorbed by paper (8) − (5) (9)	Corrected albumin value (5) + average difference ((8) − (5)) (10)	Albumin to globulin ratio from corrected values (11)
			gm. per 100 cc.	*gm. per 100 cc.*		*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	
Rabbit, concentrated	30 cc., 2, No. 5	Undiluted	10.60	7.35	2.26	7.60	7.60	0.25	7.58	2.51
		4:2	7.13	4.85	2.17		5.07	0 22	7.08	2.48
		3:3	5.29	3.53	2.00		3.80	0.27	3.78	2.50
		2:4	3.56	2.31	1.85		2.53	0.22	2.54	2.49
		1:5	1.75	1.08	1.61		1.27	0.19	1.31	2.98
Average								0.23		
Dog	15 cc., 2, No. 5	Undiluted	5.86	3.03	1.07	3.40	3.40	0.37	3.38	1.36
		4:2	3.92	1.88	0.92		2.27	0.39	2.23	1.32
		3:3	2.93	1.37	0.88		1.70	0.33	1.72	1.42
		2:4	1.92	0.77	0.67		1.13	0.36	1.12	1.40
		Concentrated	8.42	4.57	1.19		4.89	0.32	4.92	1.40
Average								0.35		
Human, multiple myeloma	30 cc., 2, No. 50	Undiluted	10.11	3.93	0.64	4.03	4.03	0.10	4.03	0.66
		4:2	6.76	2.61	0.63		2.69	0.08	2.71	0.67
		3:3	5.10	1.92	0.60		2.02	0.10	2.02	0.66
		2:4	3.44	1.22	0.55		1.34	0.12	1.32	0.63
		1:5	1.72	0.57	0.50		0.67	0.10	0.67	0.64
Average								0.10		
Human, normal	15 cc., 2, No. 5	Undiluted	7.32	4.38	1.49	4.74	4.74	0.36	4.74	1.84
		3:3	3.66	2.01	1.22		2.37	0.36	2.37	1.84
		2:4	2.45	1.22	1.00		1.58	0.36	1.58	1.82
Average								0.36		

be concluded that the paper removes a definite amount of albumin entirely independent of the actual concentration of albumin. As a further test of the validity of this calculation, when the average value for each serum is added to the determined albumin values of Column 5 and the albumin to globulin ratios are calculated with these corrected figures (Column 10), it is evident that the albumin to globulin ratios (Column 11) do not decrease with dilution, but are surprisingly constant for a given serum. We must, therefore, conclude that although we agree with the results obtained by Goettsch and Kendall, we do not agree with their explanation. It seems evident to us that the explanation lies simply in the adsorption of albumin by the filter paper.

TABLE VII

"Correct" Albumin Values on Original and Diluted Serum

Serum diluted 1:1 with 0.9 per cent sodium chloride solution. The globulin was separated by filtering through 1 sheet of No. 00 paper, 9 cm. in diameter. The albumin concentrations are in gm. per 100 cc. of the original and diluted serum. The first three 15 cc. portions of filtrate were discarded.

Human Serum No.	Dilution	4th 15 cc. portion	5th 15 cc. portion	6th 15 cc. portion	"Correct" value
32	Original serum	4.54	4.58	4.58	4.58
	1:1 serum-saline	2.28	2.29	2.27	2.28
31	Original serum	2.43	2.48	2.49	2.48
	1:1 serum-saline	1.23	1.25	1.24	1.24
30	Original serum	3.80	3.75	3.76	3.76
	1:1 serum-saline	1.91	1.90	1.92	1.91

It was next desirable to determine whether a "correct" value could be reached with diluted serum, as with undiluted serum. In the experiments of Table VII the original serum was diluted with an equal volume of 0.9 per cent sodium chloride solution. To 1 part of the diluted serum 30 parts of 22 per cent sodium sulfate solution were added. The first 45 cc. of filtrate were discarded and the following 15 cc. portions were analyzed. The results show that within the experimental error the albumin values in the diluted samples are exactly proportional to the values of the original sera.

Influence of Adsorption of Albumin by Filter Paper on Direct

Determination of Globulin—Some investigators have preferred to measure directly the precipitated globulin washed several times on the filter paper with 22 per cent sodium sulfate to remove albumin. The precipitate and filter paper are analyzed together for nitrogen.

Several experiments have indicated to us that the protein adsorbed by the filter paper cannot be easily removed by such washing. The summary of one such experiment is given in Table VIII. The albumin-sodium sulfate mixture, after passing through two sheets of No. 5 paper, had dropped from 116.3 mg. per 100 cc. of albumin to 101 mg. and after passing through two sheets of No. 50 paper, to 111.9 mg. (see Column 6). These determinations were made on 5 cc. aliquots of the filtrates, as described under "Methods." The filtrates were received in calibrated graduated tubes so that the total volume of filtrate could be measured before these aliquots were taken. With the total volume of filtrate and the albumin determination on an aliquot, the total albumin in the filtrate could be calculated. New receivers were placed under the filters and the papers were

TABLE VIII

Effect of Washing on Adsorbed Protein

15 cc. portions of a dog serum albumin-sodium sulfate mixture containing 116.3 mg. of albumin and 1.13 mg. of non-protein nitrogen per 100 cc. of solution were filtered through two sheets of Whatman No. 5 paper or two sheets of Whatman No. 50 paper. The filters were washed six times with 6 cc. portions of 22 per cent sodium sulfate solution and nitrogen determinations were made on the filtrate and washings. The results on each paper are the average of two duplicate determinations.

Two sheets of filter paper of	Recovery from 15 cc. of solution filtrate + washings					Analysis made on filtrate of 15 cc. of solution	
	Total N (1)	Albumin N (2)	Albumin (3)	Albumin calculated for 100 cc. of filtrate (4)	Albumin adsorbed by filter (5)	Albumin (6)	Albumin adsorbed by filter (7)
	mg.	*mg.*	*mg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
No. 5	2.618	2.449	15.30	102	14.3	101	15.3
" 50	2.836	2.667	16.66	111	5.3	111.9	4.4

washed six times with 6 cc. portions of a 22 per cent sodium sulfate solution. The volumes of these washings were measured and the albumin determined. The sixth washing in each case contained a negligible amount of nitrogen. Therefore, we could assume that the nitrogen of the filtrate plus the nitrogen in the washings represented the total amount of nitrogen that passed through the filter from the 15 cc. of the albumin-salt mixture. The amounts of albumin recovered from the filtrate and washings are given in Column 3. In Column 4 the albumin is calculated for 100 cc. of filtrate. These values agree with the values obtained by the determinations made directly on the filtrate without any washing (Column 6, Table VIII). They, therefore, show that in the washing the residual solution only was removed, but that the protein adsorbed by the paper was retained. This protein adsorption takes place only in the concentrated salt solutions, for when sera diluted with 0.9 per cent sodium chloride solutions in the ratio of 1:30 are filtered through the same types of filter papers, the protein concentrations are not lowered.

Modification of Method of Filtration

From these results it is evident that the filtration procedure is an important step in the albumin-globulin separation. At the present time there is no convenient substitute for filter paper. Centrifugation is not satisfactory, for many precipitates do not settle well and those that do are too easily disturbed. All the papers we have studied give clear filtrates only after several refiltrations. With the use of the hardened filter paper, less of the protein is adsorbed than with the common type of paper, and a "correct" albumin value is reached with less solution. The Whatman No. 50 paper is less retentive but allows a faster rate of filtration than the Schleicher and Schüll No. 575. However, with the procedure we recommend, the same results will be obtained with either paper, although time may be saved with the No. 50 paper. For speed and retentiveness the Munktell No. 00 is most satisfactory. Although slightly more protein is adsorbed from the first portion filtered, latter portions have always given the "correct" values.

At first it was hoped that a given size and type of filter paper would remove a definite amount of protein. This amount could then be added to the determination made on the filtrate to give the "correct" value that we obtain with the recommended method of filtration. However, many variables cause fluctuations so great that such a procedure does not seem practicable. Later, an effort was made to determine whether a correction could be added to the "minimum" value obtained after the sample had been refiltered a sufficient number of times to insure the maximum adsorption of albumin by the paper (see Table III). At the present time we do not have sufficient data to enable us to establish the constancy of this correction.

It is possible, however, so to modify the filtration procedure that the adsorption error may be avoided—by discarding the first samples of filtrate and using only the last portions for analysis. It is hard to understand why this principle has not been used before in connection with the determination of serum protein fractions, because Sørensen has repeatedly stated it; *e.g.*, Sørensen and Høyrup ((8) p. 39) state, "The first portion of the filtrate which has run through is not to be used, as—on account of the adsorbing power of the filter paper—it may have a different composition from that of the main filtrate."

The following procedure has proved satisfactory for carrying out the filtration. At least 60 cc. of the serum-salt mixture should be filtered through one sheet of a 9 cm. paper. When 2 cc. of serum are available, it is treated with 60 cc. of a 22 per cent sodium sulfate solution.

After standing for at least 4 hours, the mixture is thoroughly shaken, 15 cc. placed on the filter, and refiltered until clear. Even if the filtrate becomes clear at once, this first portion is refiltered several times in order to saturate the paper with albumin as soon as possible. It is then discarded.

A second 15 cc. portion is then filtered until clear and discarded, although in our experience the albumin value of this portion will never be more than 0.1 gm. per 100 cc. of serum lower than the "correct" value.

The next two 15 cc. portions, or any further portions, are filtered until clear and analyzed to obtain the "correct" value. The analyses are ordinarily carried out on two 5 cc. aliquots of each of these portions, giving four nitrogen determinations.

From the results we conclude that duplicate precipitations are not needed when care is taken in the initial measurement of the serum. The only value of duplicate precipitations is to detect an error in measurement of serum and to protect against accidental breakage of a flask. Therefore, when we have only a small quantity of serum, we prefer to precipitate it in one lot and filter it through one paper. In this manner good results may be obtained with only 0.5 cc. of serum, by adding to it 1.5 cc. of 0.9 per cent sodium chloride solution and 60 cc. of 22 per cent sodium sulfate solution. By addition of the saline solution the final concentration of Na_2SO_4 in the mixture is kept at 1.50 M. This procedure with 0.5 cc. of serum is satisfactory except with sera of abnormally low albumin content where the titration error is relatively increased. In general, it is an advantage to use 1 or 2 cc. portions of serum because more accurate titrations can be made and with these larger samples the increased volume of globulin which is placed upon the filter paper aids greatly in the clearing up of the filtrate.

In order to avoid the use of odd size pipettes, we often find it convenient to pipette 100 cc. of the sodium sulfate solution and add it to 1, 2, or 3 cc. of serum that have been diluted with saline to give a concentration of 1.50 M of Na_2SO_4 in the final mixture

SUMMARY

1. The experiments reported above prove that, in the presence of 1.50 M sodium sulfate solution at 37° (Howe's method), filter paper adsorbs an appreciable amount of albumin which cannot be washed out with 22 per cent sodium sulfate solution. Within rather wide limits the amount of albumin adsorbed is independent of the concentration of albumin, but is dependent upon the type and quantity of paper.

2. Refiltration of the filtrate through the same paper increases the amount of albumin adsorbed up to the "saturation" of the paper with the protein, after which further refiltrations cause no further increase in adsorbed albumin.

3. The adsorption causes an error in determination of the albumin concentration, which is magnified in calculating the albumin to globulin ratio. The error is proportionately greater when small volumes of the serum-salt mixtures are filtered (0.5 cc. + 15 cc. of Na_2SO_4 solution). When larger volumes (over 60 cc.)

are filtered in 15 cc. portions, the samples obtained after the first 30 cc. of filtrate contain a concentration of albumin that is constant, independent of the type of filter paper employed, and representative of the protein soluble in a 1.50 M solution of sodium sulfate.

4. The error due to adsorption of albumin may be avoided by the use of the modified procedure described.

Addendum—After this work was submitted for publication, the paper of Campbell and Hanna, who recommend the use of sodium sulfite as the protein precipitant, appeared in this Journal (9). We have found that the filter paper also adsorbs protein from such sodium sulfite solutions and, therefore, advise the use of our modified filtration procedure when this precipitant is used.

BIBLIOGRAPHY

1. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
2. Weech, A. A., Snelling, C. E., and Goettsch, E., *J. Clin. Inv.*, **12**, 193 (1933).
3. Howe, P. E., *J. Biol. Chem.*, **49**, 109 (1921).
4. Bruchman, F. S., D'Esopo, L. M., and Peters, J. P., *J. Clin. Inv.*, **8**, 577 (1930).
5. Howe, P. E., *J. Biol. Chem.*, **49**, 93 (1921).
6. Goettsch, E., and Kendall, F. E., *J. Biol. Chem.*, **109**, 221 (1935).
7. Robinson, H. W., Price, J. W., and Cullen, G. E., *J. Biol. Chem.*, **114**, 321 (1936).
8. Sørensen, S. P. L., and Høyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, **12**, 12 (1915-17).
9. Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, **119**, 15 (1937).

LITHOCHOLIC ACID GALLSTONES FROM HOG BILE*

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The rarity of gallstones in the hog is shown by the fact that Jolin (1) found none in approximately 450 gallbladders, while we have found only seven small stones in 6150 young hogs. When first removed these calculi were yellow, smooth, and felt somewhat waxy. After drying they became yellowish brown, hard, and brittle. They consisted mainly of lithocholic acid, probably as its calcium salt, together with small amounts of pigments. No indication was obtained of the presence of other bile acids which would have interfered with the isolation of the pure lithocholic acid. Cholesterol is present only in traces.

Lithocholic acid was first found by Fischer (2) as a minor constituent of cattle gallstones. These stones contain large amounts of pigments besides desoxycholic acid (3). Later, minute quantities of lithocholic acid were found in cattle bile (4) and in human bile (5) (1 to 2 gm. in 100 kilos of cattle bile).

The acids of hog bile consist mainly of hyodesoxycholic acid (I) (6) and 3-hydroxy-6-ketocholanic acid[1] (II) (7), but lithocholic acid (III) has not yet been found in hog bile. The presence of the acid in the gallstones suggests its occurrence in normal hog bile. The selectivity with which the acid separates from the

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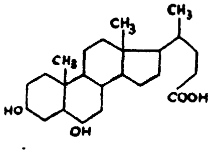
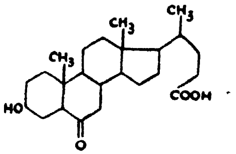
* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

[1] In the work of M. Anchel with one of us (S.) to be published later it has been found that the bile acids of hog bile contain more than 8 per cent of this keto acid.

mixture of other bile acids during stone formation may be due to the lower solubility of its salts.





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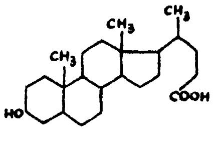

III

EXPERIMENTAL

Stone A—A suspension of 0.378 gm. of dry yellow stone powder (insoluble in acid, alkali, or organic solvents) in 10 cc. of 15 per cent hydrochloric acid and 20 cc. of ether was shaken for several hours, and then three times with 20 cc. portions of fresh ether. Except for some pigment the whole material went into solution. The aqueous layer gave a strong test for calcium. The combined ether extracts were washed with water and extracted with dilute potassium hydroxide. The residue from the ether gave a very small precipitate with digitonin (less than 0.5 mg.). The alkaline solution was acidified and the oily precipitate extracted with ether. The residue from this ether (164 mg.) was covered with low boiling petroleum ether, when crystals formed. The material was twice recrystallized from ether and twice from aqueous acetone; m.p. 184–184.5°. No melting point depression was observed when it was mixed with an authentic sample of lithocholic acid.

Analysis—$C_{24}H_{40}O_3$. Calculated. C 76.53, H 10.71
Found. " 76.45, " 10.55

Stone B—95 mg. of stone powder were treated in the same way as Stone A. 66 mg. of crude acid (m.p. 160°, not sharp) were obtained, from which 42 mg. of material, m.p. 184°, were secured.

Three other small stones (several mg. each) from different gall-bladders were investigated. The amount was not enough for the isolation of pure lithocholic acid. They all showed the same general properties as Stones A and B: insoluble in acids, alkalies, or organic solvents, but soluble when shaken with acid and ether.

SUMMARY

Several gallstones from hog bile were investigated. They consisted mainly of lithocholic acid, probably as its calcium salt.

BIBLIOGRAPHY

1. Jolin, S., *Z. physiol. Chem.*, **12**, 522 (1882).
2. Fischer, H., *Z. physiol. Chem.*, **73**, 204 (1911).
3. Küster, W., *Z. physiol. Chem.*, **69**, 463 (1910).
4. Wieland, H., and Weyland, P., *Z. physiol. Chem.*, **110**, 123 (1920).
5. Wieland, H., and Jacobi, R., *Z. physiol. Chem.*, **148**, 232 (1925).
6. Windaus, A., *Ann. Chem.*, **433**, 278 (1923); **447**, 233 (1926).
7. Fernholz, F., *Z. physiol. Chem.*, **232**, 202 (1923).

DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

X. THE METABOLISM OF BUTYRIC AND CAPROIC ACIDS*

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It has been shown in previous experiments (1, 2) that the fatty acids of the diet, whether administered in the form of neutral fats or as free fatty acids, are to a large extent deposited in the fat tissues prior to their utilization. Part of the acids was found unchanged and part had been converted into other fatty acids.

All experiments previously published by us have been carried out with the higher fatty acids containing 16 or 18 carbon atoms, all of which were normal constituents of animal depot fat. In continuation of this work we are reporting experiments with butyric and caproic (hexanoic) acids. Both are constituents of milk fat and can therefore be regarded as normal dietary components to which the animal organism is accustomed. According to the theory of β oxidation, they are both normal intermediates in the oxidative breakdown of higher fatty acids.

The deuterobutyric acid fed to the mice was prepared by hydrogenating ethyl crotonate with deuterium, and the deuterocaproic acid similarly from ethyl sorbate.

Butyric acid and caproic acid are not constituents of depot fat and are not found in it even after administration to the animal (3). In agreement with these findings, the fatty acids of our mice fed on butyric acid did not contain a trace of this substance. The problem arises whether the acid is burned immediately after absorption or stored after its conversion into such higher fatty acids as are ordinary components of depot fat.

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* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

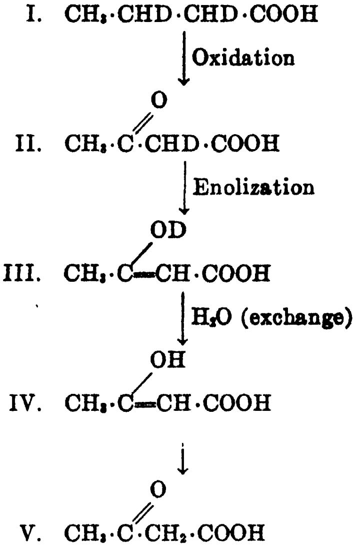
The fatty acids of mice which were fed deuterobutyric acid and killed after 8 hours had almost no deuterium in excess of the normal amount. The deuterium administered as deuterobutyric acid was found in the body fluids in the form of heavy water. Experiments were carried out to determine the mechanism by which this heavy water was formed. There exist two possibilities: It may have originated from the complete oxidation of butyric acid to carbon dioxide and water, or during a conversion of butyric acid into other substances.

In using deuterium as an indicator for biological conversion processes, one must always keep in mind that, while it is part of the molecule, this isotope serves only as a label. The biologist, in studying chemical conversions, is interested in the fate of the carbon rather than that of the hydrogen atoms. The hypothetical conversion of butyric acid into higher fatty acids means by definition the utilization of the carbon of the butyric acid for the formation of the acids in question. If during conversions the deuterium is removed, this indicator cannot directly be used for the study of such processes. If a deutero Substance A is fed to an animal and a deutero Substance B is found in the body (as in many of our earlier experiments), the deuterium content of B is proof of its origin from A. The absence of deuterium in B, however, can never be directly taken as proof for the contrary. The possibility that the deuterium was removed from the molecule during its conversion into the other substance has in each case to be considered separately.

The deuterobutyric acid given to the mice was prepared by catalytic hydrogenation of crotonic acid, the deuterium entering at the α- and β-carbon atoms.

$$CH_3 \cdot CH{=}CH \cdot COOH + D_2 \rightarrow CH_3 \cdot CHD \cdot CHD \cdot COOH$$

In this compound the deuterium is stably bound and cannot be removed from the molecule by ordinary chemical procedures. If, however, in the course of the hypothetical conversion into higher acids intermediates were formed in which the hydrogen atoms were exchangeable with the hydrogen of the body fluids, all the deuterium might be removed from the carbon chain and be found as heavy water in the body fluids, just as in the case of combustion. One such reaction might be the intermediate formation of acetoacetic acid.



$$
\begin{array}{ll}
\text{I.} & CH_3\cdot CHD\cdot CHD\cdot COOH \\
 & \downarrow \text{Oxidation} \\
\text{II.} & CH_3\cdot \overset{O}{\overset{\|}{C}}\cdot CHD\cdot COOH \\
 & \downarrow \text{Enolization} \\
\text{III.} & CH_3\cdot \overset{OD}{C}{=}CH\cdot COOH \\
 & \downarrow H_2O\ \text{(exchange)} \\
\text{IV.} & CH_3\cdot \overset{OH}{C}{=}CH\cdot COOH \\
 & \downarrow \\
\text{V.} & CH_3\cdot \overset{O}{\overset{\|}{C}}\cdot CH_2\cdot COOH
\end{array}
$$

The deuterium atom at the β position would be removed during oxidation of the butyric acid (I) to acetoacetic acid (II). The α-deuterium atom could become exchangeable by enolization (III). If in the hypothetical conversion of butyric acid into higher acids acetoacetic acid were an intermediate, deuterium could not be used *directly* for the investigation of this process.

A method which is the reverse of the first may be employed in the study of such cases. It has been shown (4) that fats, when synthesized from carbohydrates, will have stably bound deuterium, if the body fluids of the animals contain heavy water. We do not yet know at which stage of this synthesis the deuterium enters the process. A great many chemical reactions, when carried out in a medium of heavy water, must lead to such introduction: reductions, hydrations, condensations, etc. If the synthesis of stearic acid or other higher fatty acids from butyric acid is carried out in a medium of heavy water, deuterium must be expected in the synthesized fatty acid, as no route of synthesis can be formulated in which none of these reactions is involved.

For the study of such reactions the deuterium is not put into the organic molecule to be investigated, but is introduced into the body fluids in the form of heavy water. The hypothetical conversion then goes on in a medium of heavy water, and deuterium will enter the reaction. The isotope will in general then be found in a stable position in the end-product. This method may probably be used for the study of a large number of physiological reactions.

Mice were given ordinary butyric acid immediately after they had been injected with enough heavy water to increase the deuterium content of the body fluids to about 3 atom per cent. The animals were killed 8 hours after the administration of the butyric acid and the total fatty acids of the carcasses were isolated. These acids contained no deuterium in excess of the normal amount. An appreciable conversion of butyric acid into the higher acids can therefore be excluded.

The results obtained by feeding deuterocaproic acid were similar to those of the butyric acid experiments. The sodium salt was fed over a period of 3 days. The body fluids of the animals at the end of the experiment contained a considerable amount of heavy water, obviously due to oxidation of the caproic acid. The deuterium content of the fatty acids was only slightly greater than normal. This small amount of deuterium was most probably not due to the conversion of caproic acid into the higher fatty acids, but rather to the continuous synthesis from the carbohydrates of the diet during the experimental period (3 days). In any case, the amount of caproic acid deposition or conversion is extremely small, as the deuterium content of the starting material was very large (34.4 atom per cent).

EXPERIMENTAL

Preparation of Sodium Butyrate-α,β-d_2—12.1 gm. of ethyl crotonate were hydrogenated with gaseous deuterium in the presence of platinum oxide catalyst, according to the method previously described (5). No solvent was used. 1 mole of gas was absorbed. The product was distilled *in vacuo* and saponified with 7 per cent alcoholic sodium hydroxide. 50 cc. of water were added and carbon dioxide was bubbled through until the solution was neutral. The solution was evaporated to dryness and extracted with alcohol; the alcohol-soluble fraction was twice recrystallized from ethyl alcohol. The sodium salt of the acid contained 20.2 atom per cent deuterium.

Preparation of Sodium Caproate-α, β, γ, δ-d_4—7 gm. of ethyl sorbate[1] were hydrogenated with deuterium gas in dry petroleum ether, with platinum oxide as catalyst. 2 moles of gas were absorbed. The ester was saponified and the sodium salt purified by crystallization from alcohol. The substance contained 34.4 atom per cent deuterium.

Feeding Experiments with Butyric Acid

Feeding Deuterobutyric Acid and Ordinary Water—Two male mice, each weighing 20 gm., were kept on a bread diet. They were given the butyrate solution by stomach tube (ureter catheter). Mouse A received 1.0 cc. and Mouse B 0.75 cc. of 20 per cent sodium butyrate-α, β-d_2 in ordinary water. The mice were killed 8 hours after the feeding. The total gastrointestinal tract from the esophagus to the anus was removed. The tract contained a very small amount of fluid which, after treatment with hot alkali and subsequent acidification with sulfuric acid, did not develop any odor of butyric acid. The intestinal tract was discarded. The liver, spleen, kidneys, and testes were removed from the carcasses and worked up separately. A small amount of water was distilled off from the carcasses for determination of the heavy water content. Both water samples contained 0.08 atom per cent deuterium.

The carcasses were treated with alcoholic potassium hydroxide. The bones were filtered off, most of the alcohol was distilled off, and a large amount of water was added. The solution was acidified with sulfuric acid and distilled until about 20 per cent of the fluid was collected. Neither the distillate nor its ether extract had any odor of butyric acid. The higher fatty acids were extracted with ether from the residue of the distillation and purified in the usual manner. 0.983 gm. of fatty acids was obtained from Mouse A and 0.876 gm. from Mouse B. Both samples, analyzed separately, contained 0.00 atom per cent deuterium.

The organs of both mice were combined and 0.171 gm. of fatty acids was obtained, which contained 0.00 atom per cent deuterium.

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[1] The authors are indebted to Dr. Karl Meyer for supplying them with a sample of sorbic acid.

Feeding Ordinary Butyric Acid and Heavy Water—Each of two male mice (weight, 20 gm.) on a bread diet was injected with 0.6 cc. of 99 per cent heavy water. 30 minutes later the mice were given by stomach tube 1.0 cc. of 20 per cent ordinary sodium butyrate in a solution of 5.3 per cent heavy water. The mice were placed in a beaker and given bread and 5.3 per cent heavy water to drink. 6 hours after the administration of the butyric acid the mice were killed and water and fatty acids isolated from the combined carcasses as in the above experiments. The body fluids contained 3.1 atom per cent deuterium, and the fatty acids of the carcasses (1.33 gm.) contained 0.02 atom per cent.

Feeding Experiments with Deuterocaproic Acid

For 3 days two male mice weighing 20 gm. each were fed whole rye bread, to which was added sodium caproate-$\alpha, \beta, \gamma, \delta$-$d_4$. The mice had consumed about 0.4 gm. of the salt at the end of the experiment. The intestinal tract, but no other organ, was removed from the carcasses. Water and fatty acids were isolated from the bodies in the same way as in the above experiments. The water of the body fluids contained 0.49 atom per cent deuterium, and the fatty acids 0.08 atom per cent deuterium.

DISCUSSION

The experiments show that sodium butyrate, even when given in the form of a 20 per cent salt solution by stomach tube, is easily absorbed by mice. After 8 hours no residue could be detected in the intestinal tract.

The experiment furthermore indicates that the absorbed butyric acid is not deposited in the fat tissues either as such or in the form of higher fatty acids, but is probably rapidly burned. Part of the acids may have been excreted in the form of acetone bodies, though the amount of these must have been limited by the carbohydrate-rich diet.

The metabolism of butyric acid is thus different in principle from that of the higher fatty acids with 16 or 18 carbon atoms. While the higher acids are stored in the fat tissues, butyric and caproic acids are immediately and completely disposed of.

The experiments do not exclude the possibility that under different dietary conditions some of the butyric and caproic acids of the food may be stored in the fat tissues. Such a process may occur if still more excessive amounts are fed, a condition, however, which does not normally occur, as these acids usually represent only a small part of the dietary components. These acids, while constituents of some natural fats (butter), are nevertheless not fat formers in the generally accepted sense.

SUMMARY

1. The metabolism of butyric and caproic acids has been studied by administering to mice sodium butyrate-α, β-d_2 and sodium caproate-α, β, γ, δ-d_4.
2. Shortly after the administration of deuterobutyric acid it could not be detected in the animals, while heavy water was found in the body fluids. Analogous results were obtained after feeding deuterocaproic acid. The higher fatty acids of both groups of animals did not contain a significant amount of deuterium.
3. To prove that the heavy water in the body fluids had its origin in the immediate breakdown of the acids and not in the removal of deuterium from the substances during their conversion into higher fatty acids, the reverse experiment was carried out. A group of mice was given ordinary sodium butyrate and heavy water. As the higher fatty acids of these animals did not contain more than traces of deuterium, no significant conversion of butyric acid into higher fatty acids could have occurred.
4. Butyric and caproic acids are rapidly and completely disposed of by the animal and are not stored in the fat tissues, either unchanged or after conversion into higher fatty acids. They are not fat formers in the generally accepted sense.

Addendum— In addition to the study of butyric and caproic acids we have also made some studies of the metabolism of propionic acid, especially in regard to its conversion into glucose in the phlorhizinized animal. The negative results, while of some interest, do not warrant separate publication. These experiments were a necessary preliminary to the investigation of the use of deuterium for the study of the much labored question of the conversion of fat into carbohydrate by the mammalian organism. It is known that propionic acid can be quantitatively converted into glucose by the diabetic animal. The process of sugar formation as generally formulated involves a number of intermediates of which all the hydrogen is exchangeable with water. The occurrence and extent of such an exchange could be ascertained by administering to a completely phlorhizinized animal deuteropropionic acid and examining the deuterium content of the excreted sugar. We have given to totally phlorhizinized rats and a dog the sodium salt of dideuteropropionic acid ($CH_2D \cdot CHDCOONa$) containing 40 atom per cent deuterium, prepared from methyl acrylate or from allyl acetate. We have then isolated and analyzed the urinary sugar. In none of the six samples was the deuterium content of the sugar found to be greater than 0.04 atom per cent, indicating that during the conversion all, or almost all, of the deuterium had been removed from the carbon skeleton. In view of the high deuterium content of the propionic acid, the loss of deuterium is so large that it seems inadvisable to apply the deuterium method to the study of the conversion of fatty acids into carbohydrate.

BIBLIOGRAPHY

1. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **111**, 175 (1935); **113**, 505 (1936).
2. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **117**, 485 (1937).
3. Eckstein, H. C., *J. Biol. Chem.*, **81**, 613 (1929); **84**, 353 (1929).
4. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **114**, 381 (1936).
5. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **111**, 169 (1935).

THE DETERMINATION OF THIOCYANATE IN TISSUES

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(Received for publication, June 24, 1937)

It is generally accepted that when thiocyanate gains entrance into the body it diffuses only into the extracellular body fluids (1, 2). The proportionate amounts found in any one organ would therefore be a measure of the extracellular fluid in that organ and this measure could further be used in the interpretation of the distribution of other substances in organs. A study of the distribution of iodides made it highly desirable that the amount of extracellular fluid in the various organs be known, and accordingly the methods in use for thiocyanate estimation were examined. Accurate methods are available for the quantitative determination of thiocyanate in serum, urine, and saliva, but there is no satisfactory method for the determination in tissues.

In the method proposed by Corper (3) the tissue is ground with alcohol and the extract filtered. The filtrate is evaporated to dryness, the residue extracted with a small amount of alcohol, and the thiocyanate determined in this extract colorimetrically as ferric thiocyanate. Corper could recover only 70 to 80 per cent of thiocyanate when known amounts were added to tissue.

Two factors, neither of which enters into the estimation of thiocyanate in serum, must be considered in the determination in tissues. The tissue must be treated in such a way that the thiocyanate is brought completely into solution. This we have done by breaking up the tissue through digestion with alcoholic KOH. Also, the pigments normally present in tissues interfere with the final colorimetric estimation and therefore must be removed. We have found that whereas animal charcoal completely removes thiocyanate in acid or neutral solution, in alkaline solution the pigments are adsorbed by charcoal with no loss in thiocyanate.

In the method presented here the tissue is disintegrated by digestion on the steam bath with alcoholic KOH. After evaporation of the alcohol the residue is taken up with water, the resulting solution acidified with HNO_3, and the proteins removed by precipitation with tungstic acid. The pigments are removed from the filtrate by making the solution alkaline, adding animal charcoal, and boiling.

Trichloroacetic acid is unsatisfactory for the protein precipitation, since its subsequent decomposition on heating in alkaline solution forms a large amount of chloride which interferes with the ferric thiocyanate color. It has been found necessary to use HNO_3 rather than H_2SO_4, because the SO_4 ion reacts with the ferric ion to impart an interfering yellow color.

The following solutions are used.

Alcoholic KOH. Dissolve 40 gm. of KOH in a liter of 95 per cent ethyl alcohol.

10 per cent aqueous KOH.

4 N HNO_3.

10 per cent sodium tungstate.

Ferric reagent. This reagent is prepared as described by Crandall and Anderson (4). Dissolve 100 gm. of $Fe(NO_3)_3 \cdot 9H_2O$ in a liter of water and add 50 ml. of concentrated HNO_3 with shaking. Dilute to 2 liters.

Standard thiocyanate solution. Dissolve approximately 14 gm. of NaCNS in a liter of water. Mix thoroughly and standardize against $AgNO_3$ solution. Preserve in a brown bottle at ice box temperature. This solution changes slowly on standing so that occasional standardizations are necessary. Since weaker solutions of thiocyanate do not keep well, they are made from the stock solution as required.

Procedure

Place 0.5 to 1 gm. of finely hashed and thoroughly mixed wet tissue or 100 to 300 mg. of the dried pulverized tissue into a 125 ml. Erlenmeyer flask and cover with 20 ml. of the alcoholic KOH. Attach the flask to an air condenser and digest the mixture on a steam bath for 30 minutes. Then transfer the contents of the flask to a 200 ml. evaporating dish, wash the flask with alcohol, then with water to remove any residue adhering to the sides of the flask, and finally again with alcohol. The dish is placed on the steam bath and the alcohol evaporated. The residue is washed with small amounts of water into a 25 ml. graduated cylinder. Add 1 drop of phenolphthalein indicator to the solution. The solution is diluted to 20 ml. and transferred to a 125 ml. Erlenmeyer flask. Wash the cylinder with exactly 3 ml. of water and add the washings to the solution in the flask. Then neutralize with 4 N HNO_3, carefully noting the amount of acid required, and add 0.5 ml. in excess. To this acid solution is added 1 ml. of sodium tungstate solution dropwise, the flask being shaken during the addition. Sufficient water is now added to make the volume of the washing, HNO_3, and tungstic acid solution total 10 ml. Since the measurement of the final volume of 30 ml. is difficult on account of foaming, it is obtained by the sum of its components, each measured accurately. The flask is stoppered, vigorously shaken, and allowed to stand for 10 minutes. The mixture is filtered and a 25 ml. aliquot of the filtrate transferred to a 100 ml. beaker. Any turbidity in the filtrate is no objection at this point. Neutralize with 10 per cent aqueous KOH and add 0.5 ml. in excess. Then remove the pigments from the solution by adding 1 gm. of norit charcoal and heating to boiling with constant stirring. Filter while hot into a 100 ml. beaker and wash the charcoal four times with 5 ml. portions of water. If the filtrate is still colored, the above charcoal treatment is repeated.

The colorless filtrate is evaporated to about 5 ml., cooled, and to it is added 0.5 ml. of 4 N HNO_3. This solution is now quantitatively transferred to a 25 ml. glass-stoppered graduated cylinder, made up to 20 ml., and the ferric thiocyanate color developed by the addition of 4 ml. of ferric reagent. After thorough mixing, the resulting solution is compared with a standard in a colorimeter. Should a turbidity occur on the acidification with HNO_3, the ferric thiocyanate solution is filtered into the colorimeter cup. The standard is prepared by putting an amount of standard solution containing approximately the same amount of thiocyanate as occurs in the unknown into a graduated cylinder, adding 1.5 gm. of KNO_3, 0.5 ml. of 4 N HNO_3, diluting to 20 ml., and then adding 4 ml. of ferric reagent. If the volume of the standard differs from that of the unknown, then the ferric reagent is added in the ratio of 1 volume of reagent to 5 volumes of solution, and the amount of KNO_3 added is such that its concentration is equal to that in the unknown.

Although the ferric ion reaction with thiocyanate is extremely sensitive, the method as described is not applicable to tissues containing very small amounts of thiocyanate. Some unknown substance originally present in tissues or resulting from the above treatment reacts with ferric ion in acid solution to give a yellow color. This color is superimposed on that of the ferric thiocyanate, thus making it difficult to obtain accurate readings when the tissue contains less than 250 micrograms per gm. of wet tissue. However, with some experience, satisfactory values can be obtained when the tissue contains as little as 75 micrograms of thiocyanate per gm. of wet tissue. An attempt was made to eliminate the interference by the use of light filters, but without success.

Results

Table I gives a summary of the results obtained on dried liver samples to which 72 to 358 micrograms of thiocyanate had been added. The accuracy for the higher quantities represents an average error of 2 per cent for 358 micrograms and 2.7 per cent for 215 micrograms. As the amount of thiocyanate decreases, the effect of the interfering yellow color is more pronounced so that the average error for 72 micrograms becomes 8 per cent.

To show that thiocyanate present in tissues can be quantitatively recovered, the following experiment was carried out. 0.5 ml. of a solution containing 20 mg. of NaCNS was injected subcutaneously into a young rat weighing 30 gm. The animal was immediately placed in a 2 liter flask and killed in 2 hours by ether anesthesia. 500 ml. of alcoholic KOH were added and the contents digested on a steam bath under a reflux for 4 hours. The resulting mixture was filtered and a measured volume of the filtrate analyzed for thiocyanate as described above. Analysis showed that 98 per cent of the total amount injected was recovered. The undigested residue from the alcoholic KOH treatment, largely bones, was dissolved in HNO_3 and on subsequent testing found to contain no thiocyanate. The analysis of an uninjected rat was negative for thiocyanate.

Table II shows the close agreement of analyses in various tissues of a rat which had received thiocyanate, and also its relative distribution.

TABLE I

Determination of Thiocyanate Added to 200 Mg. of Dried Liver

Thiocyanate added	No. of analyses	Mean value of thiocyanate recovery	Average error
micrograms NCS		*micrograms NCS*	*per cent*
358	12	357	2
215	14	215	2.7
143	12	137	6
72	12	69	8

TABLE II

Reproducibility of Analyses on Unknown Amounts of Thiocyanate in Tissues of Rat

Tissue	Thiocyanate per gm. dry tissue
	mg.
Blood	4.26
	4.07
Lung	1.99
	2.11
Muscle	0.56
	0.59
	0.63
Liver	0.82
	0.81
	0.86
Heart	1.44
	1.54

SUMMARY

A method for the analysis of thiocyanate in tissue is described based upon an alkaline digestion of the tissue, precipitation of the proteins with tungstic acid, and removal of pigments by treatment with charcoal in alkaline solution. The thiocyanate is determined colorimetrically as ferric thiocyanate. With quantities as small as 75 micrograms the average error is about 8 per cent, with 350 about 2 per cent.

BIBLIOGRAPHY

1. Peters, J. P., Body water, Springfield, chap. 6 (1935).
2. Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., *J. Clin. Inv.*, **15,** 261 (1936).
3. Corper, H. J., *J. Infect. Dis.*, **16,** 38 (1915).
4. Crandall, L. A., Jr., and Anderson, M. X., *Am. J. Digest. Dis. and Nutrition,* **1,** 126 (1934).

THE SYNTHESIS OF ANSERINE FROM *l*-1-METHYLHISTIDINE

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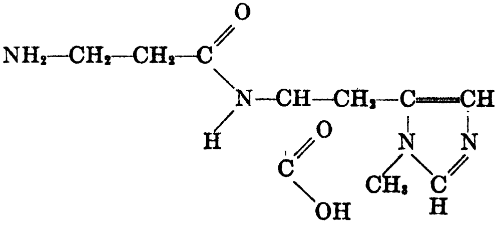
At the suggestion and through the generous cooperation of Professor Ackermann of the University of Wurzburg, the synthesis of anserine presented here was undertaken. This synthesis was made possible by Professor Ackermann's placing at our disposal his supply of *l*-1-methylhistidine (*l*-α-amino-β-(N-methyl-5-imidazole) propionic acid) which had been isolated from the hydrolytic products of anserine. The authors would like to take this opportunity of expressing their sincere appreciation for this more than generous spirit of cooperation.

The analytical approach to the structure of anserine has indicated that this muscle constituent is the β-alanyl derivative of *l*-1-methylhistidine,



After the isolation of the peptide from muscle tissue by Ackermann, Timpe, and Poller (1), it was soon suspected that anserine was a monomethylated carnosine with the methyl group attached to the imidazole ring. Linneweh, Keil, and Hoppe-Seyler (2) succeeded in isolating *dl*-methylhistidine and the α-naphthyl isocyanate derivative of β-alanine from an alkaline hydrolysis of anserine and in addition were able to demonstrate the formation of a dimethylimidazole by the alkaline decomposition of anserine in a stream of hydrogen. This demonstrated that anserine was composed of β-alanine and a methylhistidine containing the methyl group attached to the imidazole ring. Later, Keil (3) brought out the fact that the dimethylimidazole was identical with the 1,5-dimethylimidazole which had been synthesized by Pyman (4). Thus, it was clear that the methyl group was attached to the 1 position in the imidazole ring of anserine. Owing to the fact that many of the properties of anserine and carnosine were quite similar, the investigators studying the structure of the compound naturally assumed that the order of the amino acids in the peptide was analogous to that in carnosine. This assumption was finally proved correct by Keil (3) who was able to show that it was truly the β-alanyl-1-methylhistidine rather than the 1-methylhistidyl-β-alanine. This was demonstrated by the isolation of β-dinitrotoluylalanine after the hydrolysis of the compound formed from the treatment of the peptide with γ-trinitrotoluene. It was finally demonstrated by careful hydrolysis of the anserine with acid that the methylhistidine was levorotatory (5); by its analogy to *l*-histidine the assumption was made, which undoubtedly is correct, that it possessed the same spatial configuration as *l*-histidine. In this latter study the β-alanine was also isolated as the free compound. All of this analytical approach indicated, as we have mentioned above, that anserine was the β-alanyl-*l*-1-methylhistidine.

The synthesis of anserine from the methylhistidine obtained from the hydrolysis of anserine would serve to establish beyond question how the β-alanine and the methylhistidine were linked together, and would offer a crucial test of the conclusions arrived at from the analytical approach. The complete synthesis of anserine must await, however, the synthesis of *l*-1-methylhistidine.

The method which we have employed parallels quite closely that utilized in the synthesis of carnosine by Sifferd and du Vigneaud (6). Carbobenzoxy-β-alanine was converted to the azide through the acid chloride, the methyl ester, and the hydrazide, and the azide was condensed with the methyl ester of the *l*-1-methylhistidine. The resulting condensation product was saponified. In the previously reported synthesis of carnosine, the chloroform solution of the ester was shaken with a solution of

NaOH to bring about saponification. In the present synthesis, the saponification was carried out in dioxane. We have found this an advantageous modification in the synthesis of carnosine itself. The carbobenzoxy-β-alanyl-*l*-1-methylhistidine was not obtained as the free derivative but was isolated as the reineckate. After decomposition of the reineckate with pyridine, the material was catalytically reduced with palladium black in the presence of hydrogen. To effect isolation of the reduced material, the copper salt was utilized. The copper salt obtained possessed the characteristic properties of the copper salt of anserine. It gave characteristically the two forms of copper anserine, crystallizing from water in deep blue needles which upon drying in a desiccator or by the addition of alcohol became lilac in color. On evaporation of the deep blue solution of the copper salt in water, the typical lilac ring formed on the sides of the vessel. The melting point was identical with that of the copper salt of an authentic sample of anserine isolated from natural sources. Both samples melted at 238° (corrected) with decomposition and a mixture of the two copper salts showed no depression of the melting point. Analytical values for the copper salt agreed with the theoretical values. After removal of the copper in the usual way, the aqueous solution was evaporated to a syrup and by addition of alcohol colorless needles separated in clumps. The material was identical in crystalline form with the authentic sample of anserine and possessed the same melting point of 238-239° (corrected). The melting point of an equal mixture of the two compounds showed no depression in melting point. The optical rotation was slightly higher than that reported by Ackermann, Timpe, and Poller (1). The Pauly diazo reaction for the synthetic product was completely negative. The nitrate was also prepared and it likewise was identical with the nitrate of the naturally occurring anserine.

EXPERIMENTAL

l-1-Methylhistidine Methyl Ester Dihydrochloride—1.5 gm. of *l*-1-methylhistidine were dissolved in 20 cc. of absolute CH_3OH and the solution was saturated with dry HCl with heating. After 30 minutes the solution was cooled and again saturated with HCl. The CH_3OH was then removed *in vacuo*, and the syrup was redissolved in 20 cc. of CH_3OH, and the solution was again saturated with HCl. Absolute ether was added to the solution until a turbidity remained. Crystallization began at once and the mixture was then allowed to stand in the ice box. 1.7 gm. of the ester dihydrochloride were obtained. An additional 0.15 gm. was isolated from the mother liquor after concentration. The material crystallized in rods and melted at 205° (corrected). For analysis the compound was recrystallized from CH_3OH with the addition of ether and was dried *in vacuo* at 60° over P_2O_5.

$C_8H_{13}O_2N_3 \cdot 2HCl$. Calculated, C 37.51, H 5.90; found, C 37.39, H 5.95

β-Alanyl-l-1-Methylhistidine (*Anserine*)—2.1 gm. of carbobenzoxy-β-alanyl hydrazide (6) were dissolved in a mixture of 35 cc. of water, 7 cc. of glacial acetic acid, and 7 cc. of 5 N HCl, and the solution was cooled to 0°. 3 cc. of an aqueous solution containing 0.7 gm. of $NaNO_2$ were added dropwise with shaking over the course of 10 minutes. The mixture was left in the ice bath an additional 10 minutes and the azide was then extracted with $CHCl_3$, a total of 15 cc. being used in four portions. The $CHCl_3$ extract was dried with anhydrous Na_2SO_4 and filtered.

Directly into the $CHCl_3$ solution of the azide were filtered approximately 10 cc. of a $CHCl_3$ solution of the free *l*-1-methylhistidine methyl ester prepared from 1.8 gm. of the ester dihydrochloride by the method used by Fisher and Cone for histidine methyl ester (7). The mixture was allowed to stand at room temperature for 16 hours protected from moisture by a $CaCl_2$ tube, and was then evaporated to a thick syrup *in vacuo*. The residue was dissolved in 15 cc. of dioxane and 21 cc. of 1 N NaOH were added with shaking. A noticeable warming of the solution occurred. After 30 minutes the NaOH was neutralized with an equivalent amount of H_2SO_4. The solution was evaporated to dryness *in vacuo* and the carbobenzoxyanserine was extracted from the Na_2SO_4 with hot absolute alcohol. The alcohol was removed by distillation *in vacuo* and the thick syrup was dissolved in 10 cc. of water. The solution was just acidified to Congo red and a CH_3OH solution of Reinecke salt was added. A precipitate of clusters of fine, spear-like crystals was obtained. The reineckate was filtered, washed with water, and was decomposed by shaking in aqueous solution with pyridine. The pyridine reineckate was filtered and was washed with water. On the basis of 1.5 gm. of material, 8 cc. of 1 N H_2SO_4 (2 equivalents) were added to the combined filtrate and washings and the material was reduced with hydrogen in the presence of palladium black. The sulfate was removed quantitatively with $Ba(OH)_2$ and the aqueous solution was evaporated to a very small volume *in vacuo*. Addition of several volumes of alcohol caused the crystallization of 0.75 gm. of material. To effect purification of this material it was converted into the copper salt by warming with freshly precipitated $CuCO_3$ and filtering. The solution was evaporated until crystallization was well begun and 3 volumes of acetone were added. After standing in the ice box 2 hours, the precipitated copper salt was filtered. The crystals were partly dissolved in 3 cc. of H_2O. To this concentrated solution were added 10 cc. of acetone and the supernatant liquid was decanted. This process was repeated three times. The characteristic lilac-colored salt of anserine thus obtained was recrystallized from H_2O with the addition of 2 volumes of acetone.

The copper was removed with H_2S. The CuS formed a colloidal suspension and was removed by adding an equal volume of alcohol and centrifuging the mixture. The solution was evaporated to about 5 cc. and was warmed with a small amount of carbex E. The charcoal was removed by filtration and the aqueous solution was evaporated *in vacuo* until crystallization began. Addition of 5 cc. of absolute alcohol completed the precipitation. 0.55 gm. of colorless needles precipitated. This represents 29 per cent of the theory based on the methylhistidine. The compound melted with decomposition at 238–239° (corrected) and a 5 per cent solution possessed a rotation of $[\alpha]_D^{30} = +12.25°$. The value reported by Ackermann, Timpe, and Poller (1) for naturally occurring anserine is $[\alpha]_D^{16} = +11.26°$. The Pauly diazo reaction for the synthetic product was negative. The compound was dried at 120° for analysis. The dried material was very hygroscopic.

$C_{10}H_{16}N_4O_3$.	Calculated.	C 49.99, H 6.71, N 23.32
	Found.	" 49.97, " 6.52, " 23.12

The nitrate was prepared by adding nitric acid to an aqueous solution until it was just acid in reaction to litmus. The aqueous solution was evaporated until crystals appeared and several volumes of alcohol were added. The compound crystallized in colorless needles and melted at 225° (corrected). The compound was dried at 120° for analysis.

$C_{10}H_{16}N_4O_3 \cdot HNO_3$.	Calculated.	C 39.60, H 5.65, N 23.09
	Found.	" 39.43, " 5.51, " 22.87

An analytical sample of the copper salt was prepared from the free base. The characteristic copper salt was obtained, m.p. 238° (corrected). The compound was dried at 120° *in vacuo.* The dried compound was very hygroscopic.

$C_{10}H_{16}N_4O_3 \cdot CuO$. Calculated, N 17.52; found, N 17.96

SUMMARY

The synthesis of anserine has been accomplished starting with *l*-1-methylhistidine (*l*-α-amino-β-(N-methyl-5-imidazole) propionic acid). The azide of carbobenzoxy-β-alanine was condensed with the methyl ester of the *l*-1-methylhistidine. After reduction of the saponified condensation product, the resulting dipeptide was isolated as the copper salt and thence as the free crystalline compound.

Comparison of the synthetic material with an authentic sample of naturally occurring anserine demonstrated beyond question the identity of the two products.

BIBLIOGRAPHY

1. Ackermann, D., Timpe, O., and Poller, K., *Z. physiol. Chem.*, **183**, 1 (1929).
2. Linneweh, W., Keil, A. W., and Hoppe-Seyler, F. A., *Z. physiol. Chem.*, **183**, 11 (1929).
3. Keil, A. W., *Z. physiol. Chem.*, **187**, 1 (1930).
4. Pyman, F. L., *J. Chem. Soc.*, **121**, 2616 (1922).
5. Linneweh, W., and Linneweh, F., *Z. physiol. Chem.*, **189**, 80 (1930).
6. Sifferd, R. H., and du Vigneaud, V., *J. Biol. Chem.*, **108**, 753 (1935).
7. Fisher, E., and Cone, L. H., *Ann. Chem.*, **363**, 107 (1908).

EFFECT OF BILE WITH AND WITHOUT CHOLESTEROL ESTERS ON ESTERIFICATION OF CHOLESTEROL IN BLOOD PLASMA*

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It is generally assumed that cholesterol esters are not present in liver bile, but in a group of patients whose liver bile has been studied following drainage of the biliary system we encountered one patient whose bile persistently contained cholesterol in both the free and combined form (1). It was considered possible that such bile might not contain the "cholesterol esterase" of Thannhauser (2). If the esterase was absent, the effect of incubating a mixture of such bile and plasma which contains preformed cholesterol esters might be different than when an ester-free bile (normal bile) was incubated with plasma. Experiments devised to test this hypothesis were carried out and a difference in the behavior of the cholesterol esters in plasma when exposed to the two types of liver bile was demonstrated.

While our studies were being made Sperry (3) reported that the incubation of blood serum caused an increase in the combined form of cholesterol. Later he reported that bile salts inhibited this esterification in incubated blood serum (4). Normal liver bile contains considerable amounts of bile salts and we have found that such bile specimens also inhibit the esterification normally occurring in incubated blood plasma. However, the bile of the patient which contained cholesterol esters failed to have this inhibiting effect on plasma, even though it contained normal amounts of bile salts.

EXPERIMENTAL

Human and dog plasma and gallbladder and hepatic bile, obtained from biliary fistulæ, were used in these experiments. The

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TABLE I

Incubation of Normal Human Bile N_1 and Ester-Containing Bile E_1 with Normal Human Plasma A

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Hepatic bile (N_1)	0				82	82	0
Plasma (A_1)	0				224	73	151
Bile (N_1) + plasma (A_1)	0	(114)	(83)	(31)	111	86	25
Plasma (A_1)	24				222	42	180
Bile (N_1) + plasma (A_1)	24	114	73	41	117	113	4
Hepatic bile (E_1)	0				130	86	44
Plasma (A_2)	0				219	68	151
Hepatic bile (E_1)	24				130	88	42
Plasma (A_2)	24				202	39	163
Bile (E_1) + plasma (A_2)	24	146 (150)	76 (82)	70 (68)	140	72	68

Increase in combined cholesterol in normal human plasma after incubation. No increase in combined cholesterol in Bile E_1 after incubation. Normal hepatic bile + normal human plasma = decrease in combined cholesterol after incubation. Bile E_1 + normal human plasma = no change from calculated values in combined cholesterol after incubation.

TABLE II

Incubation of Normal Hepatic Bile N_2 with Normal Human Plasma J

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Hepatic bile (N_2)	0				125	125	0
Plasma (J)	0				169	55	114
" "	24				170	34	136
Bile (N_2) + plasma (J)	0	(135)	(105)	(30)	112	80	32
" " + " "	24	135	104	31	121	105	16

Increase in combined cholesterol in plasma after incubation. Normal human bile + normal human plasma = decrease in combined cholesterol after incubation.

mixture of bile and plasma was usually in the proportion of 10 cc. of bile to 3 cc. of plasma. Analyses for free and total cholesterol were made in duplicate by the procedures we have previously described (5, 6).

The human bile specimens free of cholesterol esters, which were used in these experiments, and which are designated as "normal bile," are labeled in Tables I to VI N_1, N_2, N_3, N_4, and N_5, while those from the patient whose bile contained cholesterol esters are labeled E_1, E_2, E_3, and E_4.

Table III

Incubation of Normal Human Bile N_3 and Ester-Containing Bile E_1 with Normal Human Plasma R

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Hepatic bile (N_3)	0				120	123	0
Plasma (R)	0				195	65	130
" "	24				216	58	158
Bile (N_3) + plasma (R)	24	142 (137)	105 (107)	37 (30)	121	115	6
Hepatic bile (E_1)	0				130	86	44
Plasma (R)	0				195	65	130
Hepatic bile (E_1)	24				130	88	42
Plasma (R)	24				216	58	158
Bile (E_1) + plasma (R)	24	150 (145)	76 (81)	74 (64)	144	67	77

Increase in combined cholesterol in normal plasma after incubation. No increase in combined cholesterol in Bile E_1 after incubation. Normal hepatic bile + normal human plasma = decrease in combined cholesterol after incubation. Bile E_1 + normal human plasma = no change from calculated values in combined cholesterol after incubation.

The following determinations were made: (1) free and total cholesterol in blood plasma before incubation; (2) free and total cholesterol in blood plasma after incubation at 38° for 24 hours; (3) free and total cholesterol in bile before incubation; (4) free and total cholesterol in bile after incubation. Since these data were always found to correspond with those of (3), these determinations were omitted in many instances; (5) free and total cholesterol in mixtures of bile and plasma after incubation for 24 hours at 38°.

Two sets of calculated values are given in Tables I, II, III, IV, and VI for the mixtures of bile and plasma. Those in parentheses are calculated from the values determined in (1) and (3). If the determined values for the mixture after incubation agree with this set of calculated values, it is an indication that neither esterification nor hydrolysis of the cholesterol esters of the plasma has taken place during incubation.

TABLE IV

Incubation of Normal Dog Bile and Human Ester-Containing Bile with Normal Dog Plasma

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Bladder bile (dog)	0				80	78	2
Plasma (dog)	0				149	51	98
Bile (dog)	24				80	80	0
Bile + plasma	24	(96)	(73)	(23)	96	92	4
Hepatic bile (E_2)	0				100	62	38
Plasma (dog)	0				182	58	124
Hepatic bile (E_2)	24				97	61	36
Plasma (dog)	24				181	25	156
Bile (E_2) + plasma	24	116 (119)	52 (61)	64 (57)	112	75	47

Increase in combined cholesterol in plasma after incubation. No increase in combined cholesterol in bile after incubation. Normal dog bile + normal dog plasma = decrease in combined cholesterol after incubation. Bile E_2 + normal dog plasma = slight decrease in combined form after incubation.

The second set of calculated values is determined from the values in (2) and (3). Agreement of these calculated values with the values actually determined for the mixture indicates esterification has taken place in the plasma of the mixture just as it normally would in incubated plasma alone. If the calculated value is

TABLE V

Incubation of Normal Human Bile with Ester-Containing Bile

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Bile (N_4)	0				59	59	0
" (E_3)	0				120	92	28
N_4 + "	24	89	75	14	83	68	15
" + "	24	89	75	14	85	68	17
Bile (N_5)	0				54	53	1
" (E_1)	0				133	86	44
" (N_5)	24				57	59	0
N_5 + E_1	24	94	73	21	85	71	14

Incubation of Bile E_3 or E_1 with Bile N_4 or N_5 = no change in combined cholesterol.

TABLE VI

Incubation of Normal and of Ester-Containing Bile with Plasma from Patient with Angina Pectoris

Material	Incubation time	Cholesterol, calculated			Cholesterol, found		
		Total	Free	Ester	Total	Free	Ester
	hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Hepatic bile (N_3)	0				120	120	0
" " (N_2)	0				125	125	0
" " (E_4)	0				133	113	20
Plasma (angina)	0				178	56	122
" "	24				171	35	136
Bile (N_3) + plasma	24	131 (133)	100 (135)	31 (28)	128	129	0
" (N_2) + "	24	135 (137)	104 (104)	31 (33)	125	125	0
" (E_4) + "	24	141 (143)	95 (99)	46 (44)	132	73	59

Increase in combined cholesterol in plasma after incubation. Angina plasma + Bile N_3 or Bile N_2 = decrease in combined cholesterol after incubation. Angina plasma + Bile E_4 = slight increase in combined cholesterol after incubation.

higher than the determined value, hydrolysis of esters has occurred. If the calculated value is lower than the determined value, esterification has occurred.

Results

The results are given in Tables I to VI and may be summarized as follows:

There was no change in the free and total cholesterol concentration of either normal dog or human hepatic bile, or ester-containing bile, on incubation.

The concentration of cholesterol esters in blood plasma increased on incubation and the concentration of free cholesterol decreased.

When either dog or human plasma was incubated with normal dog or normal human hepatic bile, there was no increase in the cholesterol esters. On the contrary, there was hydrolysis of the combined form in both types of plasma.

When the ester-containing bile was mixed with normal blood plasma, this inhibiting or hydrolyzing effect was absent. The amount of combined cholesterol found was approximately that which it was calculated would be found if the esterification in the blood plasma had taken place as it normally would when the plasma alone was incubated.

Normal human hepatic bile had no effect on the ester concentration of the ester-containing bile when the two were mixed and incubated.

DISCUSSION

Throughout the paper we have designated as "normal" both the dog bile and the human hepatic bile which contained no cholesterol esters. The dog bile was obtained from normal animals. The human bile was collected from patients who had some disease of the biliary tract at operation and is not, strictly speaking, normal liver bile. However, analyses of hundreds of bile specimens from patients suffering from both mild and severe types of biliary tract disease as well as the evidence of other workers (2, 7) leads us to believe that human bile, like dog bile, does not normally contain esterified cholesterol. The one case we have reported is believed to be unique in this respect.

The results of the above experiments show that there is a very marked difference between the behavior of normal bile and that of the ester-containing bile, in their effect on cholesterol esters of blood plasma. There is no doubt that some substance is present, both in dog and in human bile, which inhibits the action of the ester-forming enzyme in the blood, and also which hydrolyzes the preformed cholesterol esters. This fact would tend to support the theory of Thannhauser that bile contains a cholesterol esterase. In agreement with this concept is the fact that the patient's bile which contained cholesterol esters (and, therefore, might be lacking in the hydrolyzing enzyme) failed to have any effect on the esterification occurring normally in incubated blood serum.

Sperry, however, found that bile salts alone had this inhibiting effect on esterification of cholesterol in blood serum. Our results with normal bile could be explained on the basis of the bile salt content, but we are at a loss to explain the lack of inhibiting effect by the ester-containing bile, for it had a higher bile salt content than the so called normal human bile specimens. One must assume that some other substance present in bile may, independently of the bile salts, exert an effect on the esterification of cholesterol in plasma. Another alternative would be that the ester-containing bile, in contrast to normal bile, contained some substance which prevented the bile salts from exerting their normal inhibiting or hydrolyzing effect.

Sperry found that the esters in human serum, when the serum was incubated with bile salt solutions, reached a level below which the amount of esters did not change. In our experiments the normal bile had an equal hydrolyzing effect upon both dog plasma and human plasma, since in both the esters practically completely disappeared after incubation of the mixture. In four of five specimens of human plasma, as well as in one specimen of dog plasma, the cholesterol esters completely or nearly completely disappeared upon incubation with normal bile. This would seem further to support the hypothesis that there is something else in bile which effects esterification of cholesterol in plasma.

We are, however, at a loss to explain the results in Table V. If there is present in normal bile an esterase which hydrolyzes cholesterol esters, this should exert its effect on the ester-containing bile. However, when a normal bile and the ester-containing bile were incubated together, the normal bile had no effect on the combined cholesterol present in the other bile specimen. Calculated and determined values are in agreement.

Unfortunately we did not secure a specimen of blood serum from the patient whose bile contained cholesterol esters. This patient, aside from mild biliary symptoms, also had angina pectoris, which was the only outstanding difference between her and the other patients whose bile specimens were used for these experiments. In lieu of blood from this patient a specimen of blood from another patient with angina pectoris was studied in the hope of getting further light on the subject. It can be seen from Table VI that this blood also showed an increased esterification on incubation, that the ester was hydrolyzed by two specimens of normal bile, and that the ester-containing bile did not inhibit esterification.

SUMMARY

Incubation of plasma with normal dog or normal human hepatic bile causes hydrolysis of cholesterol esters, instead of the esterification which occurs when plasma is incubated alone. This hydrolyzing action is absent when plasma is incubated with bile containing cholesterol esters. Normal human hepatic bile had no effect on the ester concentration of the ester-containing bile.

BIBLIOGRAPHY

1. Riegel, C., Ravdin, I. S., and Rose, H. J., *Proc. Soc. Exp. Biol. and Med.*, **35**, 94 (1936).
2. Thannhauser, S. J., *Deutsch. Arch. klin. Med.*, **141**, 290 (1923).
3. Sperry, W. M., *J. Biol. Chem.*, **111**, 467 (1935).
4. Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **117**, 525 (1937).
5. Riegel, C., and Rose, H. J., *J. Biol. Chem.*, **113**, 117 (1936).
6. Rose, H. J., and Riegel, C., *J. Lab. and Clin. Med.*, **22**, 837 (1937).
7. Wright, A., *J. Exp. Med.*, **59**, 407 (1934).

ON THE NON-LABILE DEUTERIUM OF AMINO ACIDS SUBJECTED TO TREATMENT IN THE MEDIUM OF DILUTE DEUTERIUM OXIDE

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In a preliminary communication we reported some results on administering dilute D_2O to adult and growing mice and the effects thereof on the non-labile deuterium content of the protein and certain amino acids which were isolated from the tissues of these mice (1). The object of the above experiments was to ascertain the applicability of deuterium as a tool in the study of the rôle of amino acids in the synthesis of protein from the standpoint of their indispensability in nutrition. However, before conclusions are drawn regarding the significance of the presence or absence of deuterium in the amino acids which were isolated from the tissues of mice receiving D_2O, it seemed important to ascertain "by further study the manner in which deuterium could possibly enter the molecule of an amino acid" (1). We now carried out some experiments in which several amino acids of nutritionally "essential" and "non-essential" character were subjected to treatment with dilute D_2O under various experimental conditions in order to determine whether or not deuterium can enter the molecule of an amino acid by replacing hydrogen in non-labile positions.

EXPERIMENTAL

Determination of Deuterium—Combustions were carried out with 50 to 80 mg. samples in a microcombustion tube and the water collected in a glass tube packed in dry ice. For some of the earlier runs (lysine, histidine, arginine) the regular Pregl filling was employed and the tube flushed out by burning about 30 mg. of urea. For the remaining substances the lead peroxide was omitted in the filling and weighed additions of water made as necessary to bring the total amount of water to about 60 mg. Anhydrous sodium carbonate and potassium permanganate were then added and the sealed tube heated at 110° for 20 hours. The water was distilled *in vacuo* into a small ampule which was sealed off and the density of the water determined by the float method as follows:

A vessel (see Fig. 1) contained a small float (8 mm. × 0.6 mm.) in the capillary *A*, the ampule of combustion water in *B*, and an ampule of purified tap water in *C*. This vessel was evacuated and sealed at *S*, the water in *C* released by immersion in dry ice-ether which shatters the ampule, and the water distilled into *A*. The temperature at which the float remains motionless was observed (or calculated by a small extrapolation at a temperature in that neighborhood from the observed velocity of the float). The water was distilled back to *C* and this arm of the vessel sealed off. By a similar procedure the flotation temperature of the combustion water in *B* was determined and the excess in density, with respect to purified tap water, obtained from the observed temperature difference.

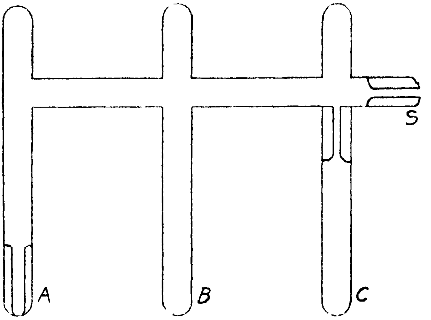

FIG. 1. Vessel employed in density measurements. The capillary *A* contained a small float, tube *B* the (diluted) sample of combustion water, and tube *C* an ampule of purified tap water for calibration of the float. *S*, outlet to pump.

Experiments with l-Cystine

Experiment 1—2.5 gm. of *l*-cystine were dissolved in 24 cc. of 20 per cent HCl which was prepared by mixing 20 cc. of 10 per cent D_2O with 27 cc. of concentrated HCl. The solution was placed in a tube, evacuated, sealed, and kept in an oven at 130°. A duplicate sample of *l*-cystine prepared in a similar manner as described above was made at the same time. One sample was kept in the oven for 4 days, the other for 7 days. At the end of the respective periods the tubes were cooled, opened, and the fluid distilled off. The residues were dissolved each in 200 to 300 cc. of distilled water, acidified, and the solutions purified by boiling with norit. The purification with norit was repeated twice. The filtrates were then adjusted to pH 4.5 with ammonia. The precipitated cystine was again dissolved in 200 to 300 cc. of water with the aid of HCl and reprecipitated with ammonia. Finally, the cystine was boiled in 200 to 300 cc. of water, and the solution cooled and filtered. The dried samples of cystine were then analyzed for optical rotation, nitrogen, and deuterium.

	4 day cystine				7 day cystine			
	N	S	$[\alpha]_D^{25}$	D_2O	N	S	$[\alpha]_D^{25}$	D_2O
	per cent	*per cent*	*degrees*	*per cent*	*per cent*	*per cent*	*degrees*	*per cent*
Theory......... .	11.67	26.69						
Found	11.67	26.66	−2.15	0.65	11.61	26.7	−2.3	0.72

Experiment 2—25 gm. of *l*-cystine were heated at 110° for 4 days in a sealed tube with 20 per cent HCl prepared as described above, with the same deuterium content. The optically inactive cystine was then isolated, and then by the procedure of du Vigneaud *et al.* (2) resolved into *dl*-cystine and mesocystine. The inactive, *dl*- and mesocystines were then analyzed for optical rotation, N, S, and deuterium as before.

	D_2O	N	S	$[\alpha]_D^{25}$
	per cent	*per cent*	*per cent*	*degrees*
Optically inactive......	0.23	11.63	26.66	−2.0
dl-Cystine dihydrochloride	0.08	8.90	20.50	−1.3
Mesocystine "	0.08	8.99	20.30	−1.7

Experiment 3—2.0 gm. of *l*-cystine were dissolved in 2 per cent D_2O with the aid of concentrated HCl, and allowed to stand at room temperature (20–22°) for 14 days. The cystine was then isolated in the manner described in Experiment 1, and analyzed for deuterium. It contained 0.05 per cent D_2O.

Experiments with Tyrosine

Experiment 4—50 gm. of casein were dissolved in 350 cc. of 2 per cent D_2O with the aid of ammonia. The pH was adjusted to 7.5 to 8.0 with phenol red as indicator, 1.0 gm. of trypsin powder was added and the mixture was allowed to stand at room temperature for 10 days with toluene as preservative. The pH was adjusted from time to time to pH 7.5 to 8.0 with ammonia. Granules of tyrosine fell out at the end of the period, glacial acetic acid was added until the pH of 5 to 6 was reached, and the solution allowed to stand 4 days, then filtered. The impure tyrosine was then dissolved in hot water, filtered, and cooled. The recrystallization was repeated three times, and the product dried *in vacuo* and analyzed.

	C	H	N	D_2O
	per cent	*per cent*	*per cent*	*per cent*
Theory	59 68	6.08	7.73	
Found	59.34	6.22	7.34	0.22

Tyrosine, isolated in the same manner from casein which was digested with trypsin in the medium of ordinary water, contained 0.02 per cent D_2O.

Experiments with Histidine, Arginine, and Lysine

Experiment 5—0.5 gm. portions of histidine hydrochloride, lysine dihydrochloride, and arginine hydrochloride were separately dissolved in 5 to 10 cc. of 10 per cent D_2O and heated at 150° in sealed tubes for 42 hours. At the end of the period each solution was evaporated to dryness, the residues dissolved in 10 to 20 cc. of distilled water, and again evaporated to dryness. The solution and evaporation of each residue were repeated three times. Then histidine and arginine were converted into flavianates, and lysine into a picrate. From these derivatives the hydrochlorides of the amino acids were obtained in the usual manner, dried *in vacuo*, and analyzed for deuterium.

	D_2O
	per cent
Histidine hydrochloride	0.58
Arginine "	0.26
Lysine dihydrochloride	0.12

DISCUSSION

The results presented above indicate that appreciable quantities of deuterium can enter the molecule of an amino acid in positions other than the imino, amino, hydroxyl, or carboxyl. Harada and Titani (3) find that as many as 3 atoms in the benzene ring of aniline hydrochloride are exchangeable. It is possible that in our experiments with tyrosine deuterium entered the benzene ring. The results with cystine, arginine, histidine, and lysine suggest that, perhaps, the exchange involved here is similar to that observed by us *in vivo* (1) and to that observed by Wynne-Jones (4) with formate, acetate, and succinate, which exchange to the extent of 8, 85, and 69 per cent respectively when treated with heavy water at 80°.

It seems that the presence of deuterium in an amino acid isolated from the tissues of animals which were receiving dilute D_2O is not necessarily an indication of the synthesis of this amino acid by the animal, inasmuch as enzymatic digestion of the food protein *in vivo* may introduce deuterium into the amino acid. The rôle of proteolytic enzymes in effecting exchange reactions with deuterium deserves close attention. The absence of deuterium in an amino acid isolated in the same manner as referred to above cannot safely be interpreted as indicating that this amino acid could not be synthesized by the animal. Such an amino acid may have had the deuterium in the molecule, but it was lost during the isolation of the amino acid, which generally involves digestion with strong acids.[1] There is no reason to doubt that the deuterium which entered the molecule of an amino acid in "stable positions" (other than imino, hydroxyl, amino, and carboxyl) can be lost when the amino acid is subjected to digestion with strong acids. Our results with cystine show that over 50 per cent of deuterium which was introduced into the molecule of *l*-cystine during racemization can be removed during the resolution.

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[1] In order to ascertain whether or not the cysteine of *p*-bromophenylmercapturic acid which is excreted by the dog upon administration of bromobenzene is derived from dietary cystine or from endogenous sources, the *dl*-cystine containing 0.65 per cent D_2O (as described above) was given to a dog which prior to that was receiving a protein-free diet (otherwise complete) for 2 weeks. 1.0 gm. of bromobenzene was then fed, and *p*-bromophenylmercapturic acid was isolated from the urine. The product contained 0.01 per cent D_2O, a value which is within the experimental error. These results cannot be safely interpreted as indicating that the dietary cystine containing 0.65 per cent D_2O was not involved in the detoxication of bromobenzene, even though the results apparently warrant such a conclusion. There is still a possibility that the heavy cystine which we fed to our dogs has lost its deuterium while in the animal organism or during the isolation of the mercapturic acid.

The work presented here suggests that the use of deuterium as a label in the study of the metabolism of amino acids in animals requires unambiguous experimental justification.

SUMMARY

1. *l*-Cystine, arginine, histidine, and lysine were shown to contain deuterium in positions other than the amino, imino, and carboxyl, when subjected to treatment with dilute D_2O and heat in the presence of HCl.

2. Enzymatic hydrolysis of casein in the medium of dilute D_2O yields tyrosine which contains deuterium in positions other than hydroxyl, carboxyl, or the amino groups.

3. The possible significance of these findings in the study of the metabolism of amino acids in animals with the aid of deuterium as a label is discussed.

The authors wish to express their gratitude to Mr. J. Alicino for the microanalytical work.

BIBLIOGRAPHY

1. Stekol, J. A., and Hamill, W. H., *Proc. Soc. Exp. Biol. and Med.*, **35**, 591 (1937).
2. du Vigneaud, V., Dorfmann, R., and Loring, H. S., *J. Biol. Chem.*, **98**, 577 (1932).
3. Harada, M., and Titani, T., *Bull. Chem. Soc. Japan*, **11**, 554 (1936).
4. Wynne-Jones, W. F. K., *Chem. Rev.*, **17**, 115 (1935).

THE EFFECT OF ALDEHYDES ON THE QUANTITATIVE DETERMINATION OF CYSTEINE AND CYSTINE

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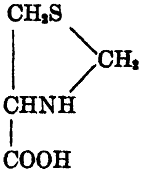
In a general study of the determination of cystine by various procedures, there was occasion some years ago to test the effect of small amounts of formaldehyde on cystine determination. It was early found that formaldehyde in short contact, 8 moles to 1 mole of cystine, had practically no action positively or negatively on the Sullivan (1) or the Okuda (2) methods but did markedly inhibit the Folin-Looney (3) and the Folin-Marenzi (4) procedures. This finding seemed to be in harmony with the early report of Folin and Looney (3) that the cystine test advocated by them was absolutely negative in the presence of sodium cyanide.

In the determination of the cystine in various purified proteins Sullivan and Hess (5) made use of the Folin-Looney results with sodium cyanide to prove that in many hydrolysates other reducing material than cystine accounted for the high Folin-Looney or Folin-Marenzi values, since a strong blue color still occurred in the presence of the cyanide.

No quantitative use could be made of the fact that the Folin-Looney cystine method was negative in the presence of sodium cyanide and other compounds were positive, since the cyanide increased the reducing value of many of the extraneous reducers tested. Among such reducers may be mentioned glyoxal, methylglyoxal, uric acid, glucic acid, ascorbic acid, all of which might be in biological solutions such as urine.

Mason (6) reported the inhibiting effect of relatively large amounts of formaldehyde upon the determination of glutathione, and cysteine in the ferricyanide oxidation procedure, and used the results to make allowance for extraneous reducers. Shinohara (7) found that formaldehyde completely inhibited the reducing power of cystine on phospho-18-tungstic acid, but did not modify the action of the various extraneous reducers tested by him. More recently, Schubert (8) and, independently, Ratner and Clarke (9) found that cysteine combines with aldehydes to make new compounds. Thus cysteine and formaldehyde give thiazolidinecarboxylic acid,



In accordance with Mason's work, the effect of a number of aldehydes was tested in this laboratory on various cystine methods.[1] Especial attention was given to formaldehyde, acetaldehyde, heptaldehyde, glyoxal, and methylglyoxal, first with short contact with cysteine and cystine respectively and secondly with long contact. The methods employed were the Sullivan (10) and the Okuda cysteine procedures, the Folin-Marenzi without sodium sulfite, and the Mason ferricyanide procedure.

The results with the various methods and a number of ratios of aldehyde to cysteine are given in Table I.

In Table I, each 5 cc. of 0.1 N HCl contained 1 mg. of cysteine (weighed as 1.3 mg. of cysteine hydrochloride) and the specified amounts of aldehyde. Shortly after being made, the solutions were tested for cysteine. It may be seen that small amounts of formaldehyde, acetaldehyde, glyoxal, and methylglyoxal, 1 mole each respectively for each mole of cysteine, have little inhibitory action on cysteine determination by the Sullivan and the Okuda cysteine methods or on the Folin-Marenzi method used without sodium sulfite. The Folin-Marenzi procedure is more inhibited by formaldehyde and acetaldehyde than is the Sullivan or the Okuda method. With glyoxal and methylglyoxal there is little apparent inhibition upon cysteine determinations except in the Mason procedure. In fact, the Mason procedure is more inhibited by acetaldehyde, glyoxal, and methylglyoxal than are the other methods. There is some question, however, as to the meaning of the Folin-Marenzi values with glyoxal. These values, especially in the higher ratios of glyoxal, seem to be a resultant of inhibition of the cysteine and positive values with the glyoxal alone. Thus 6.72 mg. of glyoxal alone give as much color as 0.67 mg. of cysteine, so it would appear that in the ratio of 14 moles of glyoxal to 1 of cysteine most of the color estimated as cysteine is due to the glyoxal.

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[1] A preliminary report of this study was given by Hess before the Division of Biological Chemistry at the Eighty-ninth meeting of the American Chemical Society at New York, April, 1935.

Table I

Effect of Aldehydes on Cysteine Determinations

Aldehyde	Ratio, aldehyde to cysteine	Methods			
		Sullivan	Okuda	Folin-Marenzi	Mason
	M	*per cent*	*per cent*	*per cent*	*per cent*
Formaldehyde	1.0:1	100			
	1.5:1	99	100	87	
	2.0:1	97	100	87	100
	4.0:1	96	100	83	80
	6.0:1	92	100	66	72
	20.0:1	91	100		29
	40.0:1	82	100		0
Acetaldehyde	1.4:1	96	100	92	76
	2.8:1	95	100	86	69
	4.2:1	86	100		56
	14.0:1	73	100	66	27
	28.0:1	66	93	50	0
Glyoxal	0.7:1	100	100	98	55
	1.4:1	86	100	98	42
	2.8:1	68	97	96	34
	7.0:1	55	90	91*	20
	14.0:1	41	83	84*	0
Methylglyoxal	1.0:1	105	100	103	53
	5.0:1	98	89	99	36
	10.0:1	90	77	97	28
	25.0:1	86	36	89	20

* Part of these values are due to glyoxal alone.

It would be rare to have these compounds in urine, blood, or other biological solution in 1:1 M relation with cysteine, at which proportion no interference occurs in dilute solutions with the Sullivan or the Okuda or the Folin-Marenzi method.

When tested on freshly prepared mixtures containing 1 mg. of cysteine per each 5 cc. of 0.1 N HCl, heptaldehyde has no inhibiting action on the Sullivan or the Okuda cysteine procedure even when the molecular ratio is 20 of heptaldehyde to 1 of cysteine. Heptaldehyde up to 5 moles to 1 has no action on the Folin-Marenzi method. At 10:1, 94 per cent of the theoretical value was found; at 15:1, 85 per cent; and at 20:1, 80 per cent.

Work with Cystine—When added to dilute solutions of cystine, 1 mg. per each 5 cc. of 0.1 N HCl, formaldehyde and acetaldehyde have, as shown in Table II, no inhibiting action in low molar relations and only slightly inhibiting action at 30 moles of 1 of cystine.

TABLE II

Effect of Formaldehyde and Acetaldehyde on Determination of Cystine

Substance	Ratio, aldehyde to cystine	Methods		
		Sullivan	Okuda	Folin-Marenzi
	M	*per cent*	*per cent*	*per cent*
Formaldehyde	2:1	99	100	42
	10:1	98	100	Negative
	20:1	96	91	"
	30:1	91	91	"
	40:1	86	80	"
Acetaldehyde	2:1	99	100	98
	10:1	99	100	89
	20:1	98	100	81
	30:1	96	96	66
	40:1	94	96	53

The Folin-Marenzi cystine procedure, however, is markedly inhibited by formaldehyde. Since glyoxal and methylglyoxal give a strongly positive reaction in the Folin-Marenzi cystine procedure, these two were omitted from the study. In the preliminary report the Folin-Marenzi cystine procedure was used for cysteine and abnormally high values were obtained when glyoxal was present.

Freshly added heptaldehyde has no inhibiting action on the Sullivan or the Okuda cystine determination in the molar relation of 5:1. Even a 20:1 ratio had little effect. In the Sullivan method, at 10:1 there is 95 per cent recovery, in the Okuda, 96 per cent; at 15:1, 93 per cent and 96 per cent; at 20:1, 92 per cent and 96 per cent. The Folin-Marenzi method at 1:1 gives 71 per cent of the theoretical; at 5:1, 58 per cent; and at 20:1, 46 per cent.

All the data given in this paper were obtained within 30 minutes of mixing the cysteine or cystine and the respective aldehydes in 0.1 N HCl. It may be said that in dilute solutions of cysteine or cystine (1 mg. in 5 cc. of 0.1 N HCl) 0.5:1 and 1:1, respectively, formaldehyde allows 98 to 99 per cent recovery of the cysteine, glyoxal 97 per cent, and methylglyoxal 95 per cent, when tested by the Sullivan procedure after 4 hours standing at room temperature. Longer contact, lessened acidity, and higher concentration of the reactants lead in the case of cysteine to the complexes, mentioned by Schubert and by Ratner and Clarke. When thiazolidine derivatives are formed, the complex naturally no longer acts as cysteine.

The effect of long contact of the materials and more concentrated solutions and different hydrogen ion concentration will be covered in another paper.

SUMMARY

On cystine determinations, the freshly added aldehydes employed have little action on the Sullivan and the Okuda cystine procedures. Formaldehyde inhibits the Folin-Marenzi cystine method markedly, heptaldehyde does so to some degree, and acetaldehyde has very little action, except in high molecular ratios.

In general the aldehydes have little inhibitory action on cysteine determinations by the Okuda method, some effect, though slight in low molar relation, on the Sullivan cysteine procedure, some inhibitory action on the Folin-Marenzi procedure as used for cysteine, and very marked retarding effect on the Mason ferricyanide procedure for —SH compounds.

BIBLIOGRAPHY

1. Sullivan, M. X., *Pub. Health Rep.*, *U. S. P. H. S.*, suppl. 78 (1929).
2. Okuda, Y., *J. Biochem.*, Japan, **5**, 217 (1925).
3. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
4. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
5. Sullivan, M. X., and Hess, W. C., *Pub. Health Rep.*, *U. S. P. H. S.*, suppl. 94 (1931).

6. Mason, H. L., *J. Biol. Chem.*, **86**, 623 (1930).
7. Shinohara, K., *J. Biol. Chem.*, **110**, 263 (1935).
8. Schubert, M. P., *J. Biol. Chem.*, **111**, 671 (1935); **114**, 341 (1936).
9. Ratner, S., and Clarke, H. T., *J. Am. Chem. Soc.*, **59**, 200 (1937).
10. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1421 (1929).

DIPHTHERIA TOXIN

I. ISOLATION AND CHARACTERIZATION OF A TOXIC PROTEIN FROM CORYNEBACTERIUM DIPHTHERIÆ FILTRATES

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The isolation of the powerful bacterial exotoxins in a state approaching purity has presented great difficulties in the past owing to the fact that the presence of large amounts of tissue extractives and peptone have been regarded as essential for growth and toxin production. The recent work of Mueller and Cohen (1–3), who have succeeded in identifying the chemical nature of the substances necessary for good growth of the diphtheria bacillus, has opened up a new approach to the study of diphtheria toxin and its production by this organism. It is now possible by suitable control of the concentration of traces of iron in the medium (4, 5) to produce toxin in high titer on a medium of entirely known composition, containing no substance of higher molecular weight than the simple amino acids (6). The availability of a method for producing large quantities of diphtheria toxin on a simple medium (7) has enabled us to isolate by ordinary neutral salt fractionation, adsorption of certain impurities on aluminum hydroxide cream, and by dialysis a protein in a high state of purity which we believe to be identical with the toxin itself.

Many previous attempts have been made to concentrate and purify diphtheria toxin from broth containing meat infusion and peptone. These have been critically reviewed in a recent paper by Eaton (8) and need only be summarized briefly here. Most of the methods used have been confined to treatment with only one or two reagents which in many instances have caused a loss of toxicity, increased flocculation time, and often a loss of antigenic power. Of the many procedures described, those involving precipitation with acids or organic solvents have invariably resulted in a profound alteration of the toxin. The work of Boivin and Izard (9), which has just appeared, on the precipitation of diphtheria toxin with trichloroacetic acid is open to this objection. Adsorption methods, neutral salt precipitation, and ultrafiltration while causing less change in toxic properties have admittedly yielded products with a higher proportion of inactive material than those obtained by acid precipitation.

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The most successful attempt to obtain diphtheria toxin in pure form has been the recent work of Eaton (8). By a rather laborious purification process, involving fractionation with ammonium sulfate, alum precipitation, adsorption of impurities with calcium phosphate or magnesium hydroxide, cadmium chloride precipitation, and dialysis, Eaton isolated from crude toxin produced on Wadsworth and Wheeler's (10) peptone medium small amounts of highly purified diphtheria toxin with a minimum loss of toxicity. He has shown that the toxin behaves as a typical heat-coagulable protein which is extremely sensitive to denaturation in acid solution. He has also demonstrated (11) that the flocculation time under controlled conditions is a sensitive index of any alteration of the toxic protein.

EXPERIMENTAL

Medium—Toxin was prepared in 15 to 20 liter lots on the gelatin hydrolysate medium previously described (7). This medium contained approximately 1 per cent of a complete hydrochloric acid hydrolysate of Eastman's purified gelatin supplemented by small amounts of methionine, cystine, tryptophane, glucose, maltose, lactic acid, the necessary salts, and alcohol-soluble accessory factors from purified liver extract. The medium contained 1.8 mg. of nitrogen per cc. and gave no precipitation with ammonium sulfate at any concentration. It was dispensed in 600 to 700 cc. amounts in soft glass Fernbach flasks (4), autoclaved, and allowed to stand at 35°, for 3 days. The flasks were then inoculated with a Park-Williams No. 8 strain (Albany No. 5 culture), incubated for 6 days at 35°, after which the organisms were filtered off through paper and the toxin collected through a Berkefeld N candle. The yield varied between 30 and 37 Lf per cc.

Purification of Toxic Filtrates—The filtered toxin was worked up in 10 to 15 liter lots during the preliminary steps of fractionation. For example, 15.3 liters of Lot GH9A (Lf = 490,000; Kf_{33} = 17[1]) were concentrated *in vacuo* to 2300 cc., with care not to allow the temperature to rise above 35° during the evaporation.[2] The concentrate was filtered and the filtrate precipitated with 1100 gm. of solid ammonium sulfate (*i.e.*, two-thirds saturation). After the mixture had stood overnight in the cold, the pink precipitate was collected on hardened paper, dissolved in distilled water, and made up to 500 cc. (total Lf = 500,000; Kf_{87} = 12). 200 cc. of saturated ammonium sulfate[3] were added and after standing in the cold, the inactive precipitate was filtered off and a further 350 cc. of saturated ammonium sulfate added. The precipitate which now formed contained most of the toxin and was collected by filtration, dissolved in distilled water, and made up to 100 cc. (total Lf = 460,000; Kf_{46} = 7). This solution was treated with 45 cc. of ammonium sulfate, filtered, the precipitate discarded, and the filtrate treated with a further 50 cc. The final precipitate was united with similar precipitates from about 100 liters of medium and made up to 165 cc. (Fraction A, total Lf = 1,800, 000, Kf_{22} = 25, precipitinogen titer = 2.8 Lf.[4]) A sample of the clear dark red solution after dialysis against dilute sodium bicarbonate and then against changes of distilled water contained 0.0005 to 0.0006 mg. of nitrogen per Lf unit. The over-all yield up to this point was about 80 to 90 per cent for most of the lots. The procedure from this stage on is summarized in Table I. The method described in Table I is not recommended for the preparation of purified toxin in high yield, but was designed to give a

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[1] Kf is taken to represent the flocculation time in minutes at 42°. The subscript denotes Lf per cc. The flocculation tests were carried out in the usual manner with 1 cc. of toxin and varying amounts of antitoxin. The same antitoxin (No. 621) was used throughout.

[2] Before concentration the toxin was adjusted to pH 7.0 or 7.4 with acetic acid.

[3] Care was taken, throughout the entire purification process, to keep the pH above 6. The saturated ammonium sulfate solutions were adjusted to about pH 7.

[4] The precipitinogen titer against the inactive diphtherial protein is given as the Lf content at the highest dilution to give a ring test after 2 hours at 25°. The preparation of this antiserum is described in a later section of this paper.

TABLE I

Fractionation of Diphtheria Toxin

Fraction	Volume	Procedure	Total Lf	Flocculation time	Precipitinogen titer, Lf
	cc.				
A	165	Dilute to 825 cc., treat with 165 cc. alumina cream,* centrifuge, and wash with distilled water. Ppt. discarded	1,800,000	Kf_{22} = 25	2.8
B, supernatant and washings	1020	Treat with 1000 cc. saturated $(NH_4)_2SO_4$. Filter. Ppt. = B_1. Add 1000 cc. saturated $(NH_4)_2SO_4$ to filtrate. Filter. Ppt. = B_2. Final filtrate discarded			
B_1, ppt.	60	To B_1 add 60 cc. water + 12 cc. alumina cream. Centrifuge and wash. Ppt. discarded. Supernatant and washings treated at volume of 150 cc. with 130 cc. saturated $(NH_4)_2SO_4$. Filter. Ppt. = C_1. To filtrate add 150 cc. saturated $(NH_4)_2SO_4$, filter, and discard filtrate. Ppt. combined as below to give C_2	580,000	Kf_{29} = 22†	3.5
B_2, "	100	To B_2 add 100 cc. water + 20 cc. alumina cream. Centrifuge and wash. Ppt. discarded. Supernatant and washings 210 cc., treated with 180 cc. saturated $(NH_4)_2SO_4$. Filter. Ppt. united with that obtained above from filtrate of C_1. United ppt. = C_2. To filtrate add 150 cc. saturated $(NH_4)_2SO_4$. Filter. Ppt. = C_3. Filtrate discarded	925,000	Kf_{28} = 17	7.0
C_1, "	50	Discarded	150,000	Kf_{27} = 70	1.7
C_2, "	100	Dialyze against two changes of 0.1% $NaHCO_3$. Volume 120 cc. Treat with 25 cc. alumina cream. Centrifuge. Ppt. discarded. Supernatant and washings 165 cc. Treat with 150 cc. saturated $(NH_4)_2SO_4$. Filter and discard small ppt. To filtrate add 100 cc. saturated $(NH_4)_2SO_4$. Filter and discard filtrate. Ppt. = D	790,000	Kf_{34} = 14	8.5
C_3, "	50	Held for future experiment	350,000	Kf_{21} = 23	10.5
D, "	60	Kept for analysis	540,000	Kf_{30} = 15	16

* Prepared according to Schmidt (12).

† Slow flocculation time. Note that the slowly flocculating material is fractionated out (Fraction C_1), the remainder having a normal Kf (Fraction C_2).

product of maximum purity even at the cost of wasting a large proportion of the material.

30 cc. of the final Fraction D in Table I were dialyzed overnight in the cold against 2 liters of 0.5 per cent sodium bicarbonate solution. The dialyzed solution was made up to 50 cc. and separated into two approximately equal fractions: Fraction E, the precipitate which formed with between 45 and 55 cc. of saturated ammonium sulfate and Fraction F, the precipitate which formed on adding a further 45 cc. of saturated ammonium sulfate to the filtrate from Fraction E. Each of the three fractions, D, E, F, was then dialyzed against two changes of 0.1 per cent sodium bicarbonate and then against three changes of distilled water. A series of analytical and biological properties was then determined on the three fractions.

General Properties of Purified Material—The final product is pale yellow in 2 per cent solution (about 6000 Lf per cc.) and nearly white when dried in the frozen state by the Flosdorf-Mudd method. The following protein tests are positive in dilute solution: biuret, ninhydrin, xanthoproteic, and Millon's. The Hopkins-Cole and the Ehrlich *p*-dimethylaminobenzaldehyde tests for tryptophane are strongly positive in 1 per cent solution, the latter giving a deep reddish violet color going to blue on standing. The Sakaguchi test for arginine is strongly positive and the Ehrlich diazo reaction gives a deep orange color. The material gives only a very weak Molisch test in 1 per cent solution and contains no phosphorus. The nitroprusside test for sulfhydryl is negative. The protein is completely coagulated by heating on the acid side of neutrality. 92 to 100 per cent of the nitrogen is precipitated from a 1.5 per cent solution with trichloroacetic acid. The toxin is completely precipitated from 1.5 per cent solution between 0.5 and 0.6 saturation with ammonium sulfate.

Analysis—The chemical composition and specific rotation of the three fractions are summarized in Table II. Except for the low rotation of Fraction F, which we are unable to explain at this time, the properties of all three fractions agree with one another as well as can be expected. Some difficulty was had in obtaining satisfactory analyses for nitrogen by the micro-Kjeldahl technique on the weighed dried material. The figures given in Table II were obtained by drying aliquots of known nitrogen content (micro-Kjeldahl) in the Flosdorf-Mudd apparatus and weighing the dried residue. This method gave 15.5 per cent nitrogen for crystalline egg albumin, in good agreement with the figures quoted in the literature.

Isoelectric Point of Purified Toxin—The isoelectric point was determined in a cataphoresis cell of the Northrop-Kunitz type. The toxin was adsorbed on washed quartz particles from a 1:2000 solution in 0.005 M sodium acetate. The direction of migration of the quartz particles coated with toxin and suspended in different 0.005 M acetate buffers was then observed (16). The pH of the buffer solutions was checked with the glass electrode. The isoelectric point was found to be at pH 4.1 ± 0.1.

TABLE II

Chemical Composition of Purified Diphtheria Toxin

Constituents	Fraction D	Fraction E	Fraction F
	per cent	*per cent*	*per cent*
Carbon	51.47		
Hydrogen	6.75		
Nitrogen*	16.1	15.9	15.8
Sulfur	0.70	0.80	0.75
Phosphorus	<0.05		
Amino nitrogen (Van Slyke)	1.12		
" " (acetone titration (13))	1.14	1.26	1.15
Tyrosine (Holiday (14))	8.0	10.9	9.5
" (Folin and Ciocalteu (15))	6.9†		
Tryptophane (Holiday (14))	1.36	1.39	1.47
" (Folin and Ciocalteu (15))	1.14		
Ash	1.36	1.59	1.35
$[\alpha]_D$‡	−36°	−40°	−24°
Isoelectric point	4.1 ± 0.1		

* Corrected for ash content.

† A precipitate formed which adsorbed some of the red color. This figure is undoubtedly low.

‡ Rotations were observed at 20° in a 2 dm. tube with 1 to 1.5 per cent solutions.

Stability of Purified Toxin—As already pointed out by Eaton

(8, 11) diphtheria toxin is extremely sensitive to denaturation in acid solution. In our experience, solutions may not be kept for any length of time at a pH more acid than 6 without undergoing change. The flocculation time or Kf is a sensitive index for even slight denaturation. Any increase in flocculation time invariably indicates a decreased toxicity. The slowly flocculating material may be separated from undenatured toxin by virtue of its diminished solubility in half saturated ammonium sulfate.

The purified preparations are also altered by shaking in dilute unbuffered solutions (17) and until this point was recognized, difficulty was had in obtaining satisfactory M.L.D. tests. The method finally adopted for determining the toxicity was to make the dilutions in 0.25 per cent Witte's peptone solution containing 0.85 per cent NaCl (Schick diluent (18)).

Toxin solutions are extremely sensitive to heat denaturation. Even at pH 8, we have observed a greatly increased flocculation time after short exposure to 45° and the toxin no longer flocculates after short incubation at 53°.

Toxicity of Purified Toxin—As mentioned above the toxin is partially inactivated by shaking in dilute unbuffered solutions. Even when diluted with Schick diluent, however, the purest fractions are found to have lost a small but definite proportion of their toxicity. This may be due to the well known phenomenon of aging or ripening of toxin, or may have been caused by the methods used in fractionating. Our fresh crude toxin generally contained close to 40 M.L.D. per Lf or flocculating unit, whereas our final fractions of purified material contained in the neighborhood of 30 M.L.D. per Lf unit and 10^7 M.L.D. per gm. The biological properties of the Fractions D, E, and F are summarized in Table III.

Atoxic Bacterial Protein—Several liters of gelatin hydrolysate medium containing 10 mg. of ferrous sulfate per liter were inoculated with the Albany No. 5 culture and incubated. No toxin was produced in the presence of this quantity of iron. The medium was filtered free from organisms, passed through a Berkefeld candle, concentrated to one-fifth its volume *in vacuo* below 35°, and brought to one-third saturation with ammonium sulfate. After the mixture had stood overnight, in the cold, the precipitate was filtered off and a further quantity of ammonium sulfate added to two-thirds saturation. Only a very small amount of precipitate formed at this point, in marked contrast to the behavior of toxic filtrates from organisms grown in the absence of inhibitory amounts of iron. The one-third saturated precipitate was dissolved in water, filtered, and reprecipitated with ammonium sulfate. Rabbits were then immunized with this inactive diphtherial protein. Precipitin tests showed that this protein was identical with that precipitated by one-third saturation with ammonium sulfate from toxic filtrates. The removal of this atoxic protein from toxic filtrates was followed by the diminution in its precipitinogen titer during the purification process, as shown in Table I. A comparison between the precipitinogen titer of the purified toxin and the original crude toxin indicates that 98 to 99 per cent of the atoxic protein has been removed during the purification as shown in Table III.

TABLE III

Activity of Purified Fractions of Diphtheria Toxin

	Fraction D	Fraction E	Fraction F	Fresh crude toxin
Nitrogen per Lf.......	0 00046	0.00045	0.00046	*Ca.* 0 05
M.L.D. per Lf*.	30	30	30	40
" " gm.*.......	1.0×10^7	1.0×10^7	1.0×10^7	
Precipitinogen titer†..	16 Lf	16 Lf	16 Lf	0.2 Lf
Kf_{30}	15	16	14	15

* To within about ±20 per cent.

† Ring test against inactive diphtherial protein rabbit antiserum. The figures are the Lf content of the highest dilution giving a definite ring test after 2 hours at 25°.

DISCUSSION

Present chemical methods for the isolation and characterization of proteins are not entirely satisfactory. Even repeated crystallization of a protein cannot be accepted as final proof of its purity. Thus Hewitt (19) has recently shown that crystalline serum albumin consists of a mixture of amorphous mucoprotein high in carbohydrate and in tryptophane and a crystalline protein of low carbohydrate and tryptophane content. In the present instance, we have purified diphtheria toxin to a point where we have been unable to alter a series of analytical and chemical properties to any significant extent by further fractionation. Since the toxic protein is rapidly denatured at pH levels below 6 (*i.e.*, in solutions more acid than 2 pH units above the isoelectric point), it seems improbable that the toxin will be obtained in definite crystalline form for some time. Nevertheless, purified diphtheria toxin, precipitated by dialysis against two-thirds saturated ammonium sulfate buffered with phthalate at pH 6.1, when stirred forms silky swirls which may possibly consist of very small crystals. Under the microscope the precipitate appears as very small particles of uniform size, but no definite crystal angles could be assigned.

While the data given in Table II must not be regarded in any way as final or as absolute criteria of purity, it is felt that the figures do serve to characterize and differentiate diphtheria toxin as a protein, although no chemical evidence is available at the present time to account for its remarkable toxicity. We believe that the most convincing evidence for the purity of our preparations is the close check between the nitrogen per Lf value for diphtheria toxin obtained from a quantitative study of the flocculation reaction and that found by isolation as described in this paper. The quantitative study of the flocculation reaction, already reported elsewhere (20), in which a method independent of the purity of the toxin employed was utilized, indicated 0.00046 mg. of nitrogen per Lf unit of diphtheria toxin. This figure is the same as that found for our purest preparations within the limits of experimental error. It is also close to the value found by Eaton (8) for toxin isolated from peptone medium. Eaton's material contained 0.0005 to 0.0006 mg. of nitrogen per Lf unit, of which 0.00045 to 0.0005 mg. was precipitable by trichloroacetic acid.

Our material differs from that reported by Eaton in two respects. Eaton reports on the basis of qualitative tests that his toxin contains no sulfur and no tryptophane. All three of our fractions contain close to 0.75 per cent sulfur by analysis. There can be no doubt that this sulfur is organic, since no sulfate was precipitated with barium chloride from the dialyzed toxin and over 80 per cent of the sulfur found was volatile on combustion. The absence of tryptophane in Eaton's preparations is difficult to explain. All our fractions give strong Ehrlich and Hopkins-Cole tests for this amino acid and all show 1.4 to 1.5 per cent tryptophane by the ultraviolet absorption method of Holiday (14). Finally, as a check on the latter method we have analyzed 140 mg. of sample by the Folin-Ciocalteu (15) method and found 1.14 per cent tryptophane. On the basis of its tryptophane content, diphtheria toxin has a minimum molecular weight between 14,000 and 18,000.

Some preliminary measurements of the thickness of monomolecular films of purified toxin on barium stearate (21) have been made by Dr. Eliot F. Porter of the Biological Institute, Harvard University, in collaboration with Dr. Irving Langmuir at the General Electric Laboratory in Schenectady. It is of interest that these preliminary studies give a film 36 Å. thick[5] (22), a finding which, assuming a spherical molecule under these conditions, suggests that the toxic protein is of relatively small size and indicates that its molecular weight is probably about 17,000.

SUMMARY

A toxic protein has been isolated from crude diphtheria toxin and has been characterized. Evidence has been presented that this protein is essentially free from other proteins elaborated by the diphtheria bacillus and from constituents of the medium. It would appear from the evidence presented that the protein is identical with diphtheria toxin. The toxin behaves as a typical heat-coagulable protein which is extremely sensitive to denaturation by solutions more acid than pH 6 and by moderate temperatures. The purest preparations contain about 16 per cent nitrogen, 0.75 per cent sulfur, 9 per cent tyrosine, and 1.4 per cent tryptophane. The isoelectric point is 4.1 and the specific rotation close to $-40°$. Approximately 0.0001 mg. of the dried material is sufficient to kill a 250 gm. guinea pig in 5 days.

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[5] The same method gives 50 Å. for the diameter of the egg albumin molecule (22) of which the molecular weight is well known to be 35,000.

the ultraviolet absorption spectrum analyses of diphtheria toxin, to Mr. William Stein for valuable assistance in determining the isoelectric point, and to Mr. William Saschek for making the sulfur, carbon, and hydrogen analyses. He is also indebted to Dr. Elliott S. Robinson and Dr. F. D. Hager of this laboratory for valuable suggestions and criticism.

BIBLIOGRAPHY

1. Mueller, J. H., *J. Bact.*, **29**, 515 (1935).
2. Mueller, J. H., *J. Biol. Chem.*, **119**, 121 (1937).
3. Mueller, J. H., and Cohen, S., *J. Bact.*, in press (1937).
4. Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, **17**, 335 (1936).
5. Pappenheimer, A. M., Jr., *Brit. J. Exp. Path.*, **17**, 342 (1936).
6. Pappenheimer, A. M., Jr., Mueller, J. H., and Cohen, S., *Proc. Soc. Exp. Biol. and Med.*, **36**, 795 (1937).
7. Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, **18**, 239 (1937).
8. Eaton, M. D., *J. Bact.*, **31**, 367 (1936).
9. Boivin, A., and Izard, Y., *Compt. rend. Soc. biol.*, **124**, 25 (1937).
10. Wadsworth, A., and Wheeler, M., *J. Infect. Dis.*, **55**, 123 (1934).
11. Eaton, M. D., *J. Immunol.*, **30**, 361 (1936).
12. Schmidt, S., *Ann. Inst. Pasteur*, **46**, 202 (1931).
13. Linderstrøm-Lang, K., *Compt.-rend. trav. Lab. Carlsberg*, **17**, No. 4 (1928).
14. Holiday, E. R., *Biochem. J.*, **30**, 1795 (1936).
15. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
16. Abramson, H. A., Electrokinetic phenomena and their application to biology and medicine, American Chemical Society monograph series, New York (1934).
17. Glenny, A., Pope, C. G., Waddington, H., and Wallace, V., *J. Path. and Bact.*, **28**, 463 (1925).
18. White, B., Bunney, W. E., and Malcolm, W. G., *J. Immunol.*, **22**, 93 (1932).
19. Hewitt, L. F., *Biochem. J.*, **30**, 2229 (1936).
20. Pappenheimer, A. M., Jr., and Robinson, E. S., *J. Immunol.*, **32**, 291 (1937).
21. Langmuir, I., Schaefer, V. J., and Wrinch, D., *Science*, **85**, 76 (1937).
22. Langmuir, I., and Schaefer, V. J., *J. Am. Chem. Soc.*, **59**, 1406 (1937).

THE USE OF DIFFERENT MEASURES OF REACTION VELOCITY IN THE STUDY OF THE KINETICS OF BIOCHEMICAL REACTIONS

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Examination of the considerable body of data which has been gathered on the effect of various factors, such as pH, catalyst concentration, temperature, viscosity, etc., on the velocity with which substances of biochemical significance react, shows that the term reaction velocity has had different meanings in the hands of various investigators. In general it has been employed in three different senses: (*a*) extrapolations or averages of values *calculated* as reaction constants, usually monomolecular constants, (*b*) the amount of reactant changed in a given time during the first part of the reaction, (*c*) reciprocals of the times required to effect a given change.

Since, as will be pointed out more fully later, many generalizations of wide significance have been and are being drawn from such data, it is of fundamental importance to determine the circumstances in which each of the above measures may correctly be used to represent the velocity of reaction. The concern of the present paper is, therefore, to consider the description of the rate of reaction by means of reaction constants, to point out the general relationships between the reaction constant and the other measures of reaction velocity, and to describe the conditions of applicability which proceed as a consequence of these relationships. These conditions are illustrated by reference to the phosphatase hydrolysis of sodium β-glycerophosphate and more generally to the velocity of other enzymic reactions, since it is such reactions the kinetics of which have been most intensively studied. Finally, the evaluation of data which have been gathered by investigators employing various measures, or of conclusions based on such data, is briefly indicated.

EXPERIMENTAL

Full details with regard to the methods of preparing phosphatase extracts and the measurement of their action on sodium β-glycerophosphate are given in previous papers (1, 2).

Theoretical

The assumption which underlies formulations of the relation between the instantaneous rate of reaction and the concentrations of the reactants at any moment is the law of mass action: that the rate at which a substance takes part in a reaction is proportional to the concentration raised to that power which represents the number of molecules taking part in the reaction. Such formulations are made for simultaneous as well as isolated reactions, and for heterogeneous as well as homogeneous systems. The integration of such formulations leads to expressions which contain the supposed constant of proportionality, or reaction constant, and concentration and time terms which are experimentally determinable. The finding that k, the value calculated from any of these expressions is, *within experimental error,* indeed constant for any values of x, y . . . (concentrations of reactants at any moment), a, b . . . (original concentrations of reactants), and t (time) justifies the original assumption.

But it is necessary to consider closely what is meant by the phrase, *within experimental error.* If it were possible to know the *true* values of x, y . . ., a, b . . ., and t, substitution of these in the various integral expressions would, of course, reveal immediately whether the mass law assumption underlying the particular formulation was justified. However, as Reed and Theriault (3) have pointed out for the monomolecular expression, the inevitable constant errors of observation introduce trends in the values of the reaction constant computed from the integral expressions, though such trends may occasionally be masked by the accidental errors. Reed and Theriault have, in the case of the monomolecular expression, circumvented this difficulty by developing a statistical treatment of the reaction velocity data which "permits of the ready derivation both of the experimental constants and of their precision values, proper allowance being made for constant errors."

It follows from the expressions for the various instances of the mass law assumption, that when the initial concentrations of the reactants (assuming *true* values for these, of course) are held constant as a given condition, *e.g.* temperature, is varied, the reaction constant is a direct measure of the instantaneous rate of change for any and every given stage of the reaction. Thus, in the case of the monomolecular expression, as a given condition is varied,

$$\left(\frac{dx}{dt}\right)_A = k_A(a_1 - x)$$

$$\left(\frac{dx}{dt}\right)_B = k_B(a_1 - x)$$

For any value of $x = x_1$

$$\frac{\left(\frac{dx}{dt}\right)_A}{\left(\frac{dx}{dt}\right)_B} = \frac{k_A}{k_B}$$

The relation between a reaction constant, k, the *true* concentration of reactant changed, x, and the *true* time, t necessary for this change may be generally expressed as

$$t = \frac{1}{k} \times f(x) \tag{1}$$

where x is a continuous function of t. If it is assumed that the form of the function does not change as a given condition is varied, then for any two variants

$$t = \frac{1}{k_A} \times f(x) \tag{2}$$

$$t = \frac{1}{k_B} \times f(x) \tag{3}$$

For a given value of t equal to t_1, Equations 2 and 3 become $t_1 = (1/k_A) \times f(x_A)$; $t_1 = (1/k_B) \times f(x_B)$. Since the left-hand members of the two equations are equal, then

$$\frac{k_A}{k_B} = \frac{f(x_A)}{f(x_B)} \tag{4}$$

On the other hand, for a given value of x equal to x_1, Equations 2 and 3 become $t_A = (1/k_A) \times f(x_1)$; $t_B = (1/k_B) \times f(x_1)$. After division and transposition, the following expression is obtained

$$\frac{k_A}{k_B} = \frac{1/t_A}{1/t_B} \tag{5}$$

In a reaction of a given type, then, the ratio of the reaction constants at different variants of a condition is equal to the ratio *of the functions of the amounts* changed in a given time, or the ratio of the reciprocals of the time required to effect a given change.[1]

Use of Values Calculated As a Reaction Constant As a Measure of Reaction Velocity—The constant calculated from the monomolecular expression, $k = (1/t) \ln (a/(a - x))$, has been used very widely as a measure of reaction velocity. In some reactions, such as the frequently studied acid hydrolysis of sucrose, its use has been held particularly appropriate. In this reaction many investigators have found that k apparently does not vary with change in the initial concentration of sucrose and, at a given sucrose concentration, is apparently constant throughout the entire time course of the reaction (5). Occasionally, however, some have found the value calculated from the monomolecular expression to rise slightly during the course of the reaction (6) and have attributed this slight rise to be due to increased activity of the sucrose or of the hydrogen ion catalyzing the reaction (3, 6). However, Reed and Theriault found, upon statistical treatment of Pennycuick's data (6), which, according to that author, had shown a rise of 3 to 5 per cent in the value of k during the course of the reaction, that there was instead very close conformance to the equation of the monomolecular type. The deviation which Pennycuick noted was apparently due to the constant errors of observation.

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[1] This latter relation was observed by Osterhout (4) to hold for reactions of the first or higher order and for consecutive and autocatalytic reactions. It must be recognized, however, that the use of the reciprocal of the time required to effect a given change as a measure of reaction velocity is, in functions the form of which is known, experimentally convenient but not necessary. It is in unknown functions that the use of this measure becomes necessary. In such cases the assumption, implicit in Osterhout's treatment, must be made quite explicit: that the form of the function does not change with variation in condition. This will be discussed more fully later.

When values for k, calculated from the monomolecular expression, have been used as a measure of reaction velocity in enzyme reactions, defections from constancy, *usually of much greater magnitude* than those noted in the acid hydrolysis of sucrose, have been obtained. Though undoubtedly some of this defection, in a given instance, is due to the existence of constant errors of observation, it can be shown that by far the greater portion of such defection, and the variations in such defections noted by different observers, are due to the physicochemical conditions of the reaction.

In the acid hydrolysis of sucrose there is very little evidence to complicate the simple mass law assumption that the instantaneous rate of reaction is proportional to the concentration of sucrose present at any moment. The constant, calculated from the monomolecular expression, appears independent of the initial concentration of sucrose as this is varied; glucose, one of the reaction products, does not retard the action. These conditions do not hold, however, for the invertase hydrolysis of sucrose (7). Moreover, the presence of different accompanying substances in the enzyme extract and the change of pH in the case of certain preparations markedly, and beyond experimental error, alter the form of the time-change curve, and if values calculated from the monomolecular expression are used to describe the course, the magnitude or the direction of the trend of these.[2]

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[2] These considerations as well as others to be presented make it apparent that the question of the monomolecularity of the action of invertase, phosphatase, or of many other enzymic reactions is, in contrast to the acid hydrolysis of sucrose, hardly a pressing one. In the latter case, the results of different investigators shuttled back and forth between the finding of monomolecularity and of slight but definite deviations from it; the situation demanded a statistical treatment of the reaction velocity data to determine whether the trends noted were due to slight deviating influences, such as increase of hydrogen ion activity, or to constant errors of observation. But for enzymic reactions, the known retardant effects of reaction products; the

Thus, Willstätter, Graser, and Kuhn (11) found, with a given preparation of invertase, that at pH levels below 6.0, the increase in the value of *k* calculated from the monomolecular expression was about 30 per cent during the course of the hydrolysis. At levels above pH 6.5 the value similarly calculated decreased about 30 per cent. For this reversal in the trend of *k* to be due to a constant error of observation, it would be necessary to assume that the increment of OH^- ions could, in addition to the alkali used to mutarotate the hydrolysis samples before the final polarimetric readings, have an effect on those readings. The studies of Vosburgh (12) on the influences affecting polarimetric readings make the entertainment of this possibility extremely difficult.

Nelson and his coworkers found that *k*, calculated from the monomolecular expression, rose markedly during the course of the hydrolysis of sucrose by invertase. Nelson and Hitchcock (13) devised, using the method of least squares, an empirical equation to fit the observed data. Of the eight preparations of invertase which they used, six gave values for the empirical reaction constant, *n*, which were indeed constant, within an average

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obvious failure of the constant, calculated from the monomolecular expression and approximate as it may be, to be independent of the initial concentration of substrate; the possible inactivation of enzyme during its action; the presence of accompanying substances in the enzyme extract affecting such inactivation make the simple assumption of the monomolecular form of the mass law impossible. At the very most, one might make the assumption that, within certain narrow limits and for some preparations of a given enzyme, the resultant effect of all of these factors is such that, fortuitously, the rate of change is proportional to the concentration of substrate. In the absence of the quantitative evaluation of the effect of each of these factors, it hardly seems of moment to determine the precision of such circumscribed data by the statistical procedure of Reed and Theriault. Moreover, where data of precision and of some generality have been obtained, like those of Nelson and his coworkers (7) on invertase, it is evident that the departure from monomolecularity is marked and well outside any experimental error. It is to be noted that there have been several attempts to formulate enzymic reactions, when departures from the simple monomolecular mass law assumption are taken into consideration. Such, for example have been the equations of Van Slyke and Cullen on urease (8), of Northrop on trypsin (9), and of Barron on α-ketonoxidase (10). The demonstration of the generality of such formulations would, of course, make most appropriate the application of a statistical procedure to the reaction velocity data as a criterion of the reliability of the formulation.

deviation of less than 0.7 per cent from the mean, throughout the entire course of the reaction. Two preparations, however, yielded data which showed a downward trend in the value of n, beyond the 0.7 per cent employed to denote the effect of errors of observation. Nelson and Hollander (14) showed that these preparations were more unstable and found for one, though not for the other, that the addition of sodium chloride or yeast gum eliminated the downward trend. This, then, is another instance where change in the form of a function describing the time course of a reaction cannot be attributed to an alteration in the magnitude of the constant errors of observation, but must be attributed to a change in the physicochemical conditions of the reaction.

The many investigators who have employed the values for the constant, calculated from the monomolecular expression, as a measure of reaction velocity have, to characterize the particular reaction, extrapolated such values to 0 time, or taken the average of several of them, either at the beginning of the time course or later on. It may be seen from the preceding discussion that such a procedure tends to obscure the fact that the value calculated as the monomolecular constant may change in different ways under different conditions such as pH, temperature, etc., and thus fails to assign the proper rôle to such a change in the measurement of the reaction velocity.

This inadequacy of the value, calculated from the monomolecular expression, as a measure of reaction velocity may be illustrated strikingly in the case of the phosphatase hydrolysis of sodium β-glycerophosphate. In Table I is shown, as typical of many such experiments, the action of a cattle bone phosphatase, Preparation CBH-3, (*a*) without the addition of magnesium or glycine, (*b*) in the presence of 0.00625 M added glycine, and (*c*) in the presence of 0.00625 M added glycine and 0.009 M added magnesium ion. When neither added magnesium nor glycine is present, the value calculated as the constant from the monomolecular expression falls very rapidly. Thus, its value at 35 minutes when 1.30 per cent of the phosphorus has been liberated is 0.36×10^{-3}; at 268 minutes, when 6.83 per cent has been liberated, the value is 0.27×10^{-3}, and when 26.1 per cent has been liberated, the value has fallen to 0.07×10^{-3}. In the presence of 0.00625 M glycine, the value calculated as the constant from the monomolecular expression does not fall so rapidly. Thus, it is 0.36 × 10^{-3} at the beginning of the reaction, and is 0.16 × 10^{-3} when 22.6 per cent of the phosphorus has been liberated. In the presence of magnesium and glycine the value calculated as the monomolecular constant, k, is about 0.72 × 10^{-3} and does not decrease during the part of the reaction (the first two-thirds) followed. (*No special significance is to be attached to this apparent constancy;* in connection with the other results, it is merely an indication of the change in the form of the time-action curve.)[3]

TABLE I

Effect of Glycine, and of Magnesium and Glycine on Change in Value Calculated As Monomolecular Reaction Constant, during Course of Phosphatase Action

Temperature, 25°; concentration of cattle bone phosphatase, Preparation CBH-3, 12.5 per cent by volume of hydrolysis mixture; concentration of sodium β-glycerophosphate, 0.0127 M; optimal pH, 9.1; k as given is $(1/t) \times \ln (a/(a-x)) \times 10^3$.

No addition			Addition of 0.00625 M glycine			Addition of 0.00625 M glycine, 0.009 M magnesium		
Time	Amount hydrolyzed	k	Time	Amount hydrolyzed	k	Time	Amount hydrolyzed	k
min.	*per cent*		*min.*	*per cent*		*min.*	*per cent*	
35	1.30	0.36	35	1.30	0.36	16	1.18	0.75
75	2.43	0.35	75	3.01	0.41	43	2.95	0.70
150	3.94	0.28	150	5.06	0.35	109	7.65	0.73
268	6.83	0.27	268	9.20	0.36	194	12.5	0.69
442	7.82	0.19	442	11.0	0.27	402	26.4	0.76
1465	13.9	0.10	1465	22.6	0.16	1428	64.6	0.70
4450	26.1	0.07	4450	38.0	0.11			
8700	40.2	0.06	8700	48.9	0.08			

In previous studies (1) it was shown that glycine or other α-amino acids, either added or present as products of autolysis, prevent the inactivation of phosphatase during its action on the substrate. The use of the value, calculated as the monomolecular constant and extrapolated to 0 time, to compare the rate of action of two phosphatase preparations containing different concentrations of α-amino acids would therefore give an incorrect representation of the rate of action *during* the hydrolysis.

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[3] Calculations from other data (15) do not lead to values of k which are constant throughout the entire course of the reaction; the effect of the presence of magnesium and glycine in preventing the marked decrease in the value of k is, however, evident in such data.

There is, however, another type of error incurred by the use of values calculated as the monomolecular constant. Table II shows a pair of devised bimolecular reactions one of which proceeds, by definition, at twice the rate of the other; these reactions are intentionally calculated as monomolecular and show, therefore, decreasing values for the monomolecular constant. Because of the fact that the value so calculated decreases at different rates in the two reactions, comparison at a given time introduces an error. Thus at 20 minutes the ratio of the values is 0.0176:0.0096 = 1.83 or 0.17 less than the actual ratio, 2.00, of the rates; at 40 minutes the ratio is 1.72 or 0.28 less than the actual ratio. The gravity of the error introduced by such procedure depends, in the particular case, upon how rapidly the value calculated as the

TABLE II

Demonstration of Error Incurred by Employing Pseudomonomolecular Reaction Constant As Measure of Reaction Velocity

A pair of bimolecular reactions, A and B, one of which is proceeding at exactly *twice* the rate of the other (k for $A = 0.000210$, k for $B = 0.000105$) is solved for values of the amounts changed, x, at various times, t. These values of x are then used in calculating the pseudomonomolecular reaction constant, $k = (1/t) \ln (a/(a-x))$. The values so calculated decrease during the course of the reactions. The last column shows the ratio of the pseudomonomolecular constants for the two reactions at various points in their time course.

Time	Pseudomonomolecular reaction constant		Ratio of pseudomonomolecular reaction constants
	A	B	
min.			
5	0.0202	0.0103	1.96
10	0.0191	0 0101	1.89
20	0.0176	0.0096	1.83
30	0.0163	0.0091	1.79
40	0.0151	0.0088	1.72
60	0 0137	0 0081	1.69
80	0.0123	0.0075	1.64

monomolecular constant decreases and at the time in the course of the action that they are compared. In some data, the error is within experimental limits; on the other hand, there are data in which the error may be considerable (16).

To summarize, the use of the reaction constant parameter as a measure of reaction velocity is justified (*a*) when there is no evidence to contradict the particular mass law assumption made and (*b*) when the value of the parameter, k, calculated from the integral expression derived from the assumption is indeed constant during the course of the reaction, proper allowance being made for experimental errors. On the other hand, the use of the parameter, k, when it is calculated from expressions based on unjustified mass law assumptions may be expedient, but is usually approximate and, in certain cases, is erroneous.

Use of Amount of Reactant Changed in a Given Time during the First Part of the Reaction As a Measure of Reaction Velocity—It has been shown, in the course of this paper, that comparison of the reaction velocities, as a given condition is changed, may be correctly made by using *not the ratio of the amounts changed in a given time, but of the functions of these amounts.* However, in a reaction of 0 order, $x = kt$, the ratio of the functions of the amounts changed is, in effect, the ratio of the amounts themselves; the latter may, therefore, be correctly used in the comparison of reaction velocities.

A considerable number of enzymic reactions, under the conditions used in their investigation, are of 0 order during the initial stages. Thus the oxidation of pyruvic acid by α-ketonoxidase (10) is of this order for about the first 50 per cent of the action on the substrate; the hydrolysis of sucrose in the presence of yeast invertase is of 0 order during the first 10 to 15 per cent cleavage of the sucrose (13). It cannot be assumed, however, that all enzymic preparations will show such a portion of their action initially to be of 0 order. It was pointed out in previous studies (1) that the action of certain bone phosphatases or of dialyzed kidney and intestinal phosphatases is of this order during the first 1 or 2 per cent of the hydrolysis of sodium β-glycerophosphate; in the presence of optimal concentrations of glycine (0.00625 M) and of magnesium (0.009 M) the portion of the hydrolysis which shows a constant velocity increases to about 10 per cent of the cleavage of the glycerophosphate.

Though some investigators, in studying the factors influencing the velocity of enzymic reactions, meticulously confine themselves to that initial portion of the reaction where the rate of change of the substrate is constant, many investigators waive preoccupation with the form of this part of the time-change curve. In some instances, there is a somewhat arbitrary designation of a time interval from the beginning of the reaction during which the amount changed is measured, regardless of the extent of the action which this amount represents; in other instances, the time interval is chosen to correspond to a small change, but there is no demonstration that the particular reaction being investigated is indeed of 0 order during this interval.

The extent of the error incurred by using the amount changed in a given time as measure of the reaction velocity depends on the degree of deviation from a 0 order reaction; that is, upon how large the relative negative acceleration of the instantaneous rate of reaction, dx/dt, has been up to the time taken for measurement. It may be shown, for instance, that in a pair of devised monomolecular reactions, one of which is proceeding by definition *at twice the rate* of the other ($k = 0.0230$ and $k = 0.0115$, respectively) the ratio of the amounts changed by the end of the first 10 minutes is 20.8 to 10.8 per cent, or 1.94; the ratio of the amounts changed at the end of the first 20 minutes is 36.8 to 20.6 per cent, or 1.78, 11 per cent less than the actual ratio of the reaction velocities. In bimolecular reactions proceeding at about the same rate, the discrepancy between the ratio of the reaction constants and that of the amounts changed in a given time is somewhat greater. Since the decrease of the rate of change in the types of reactions just mentioned is approximated in many enzymic reactions, the error incurred by using the amount changed in a given time as measure of the reaction velocity is of a similar order.

Many examples of an incorrect use of this measure of reaction velocity may be noted in the literature, current as well as past; a recent example may be cited in illustration. In Table III are shown some kinetic studies on the cleavage of guanine desoxyriboside by nucleosidase (17). It may first be noted that the value calculated by the author as the monomolecular constant decreases quite markedly during the course of the reaction. The effect of the concentration of nucleosidase on the velocity of reaction is studied by comparing the amounts of guanine desoxyriboside hydrolyzed in 1 hour. No evidence is offered that up to this time the reaction is, at the various enzyme concentrations, of 0 order. In fact, the only data available on the course of the reaction indicate quite strikingly that the contrary holds. The use of this measure of reaction velocity is, under the circumstances, incorrect and it is not surprising that the author does not obtain direct proportionality between enzyme concentration and reaction velocity, a relationship which, it will be noted later, seems to hold generally for enzymes without any well supported exceptions.

TABLE III

Cleavage of Guanine Desoxyriboside by Nucleosidase. Data of Klein (17)

Temperature, 37°; 50 mg. of guanine desoxyriboside in a total volume of 50 cc. Extent of action measured by titration with 0.01 N iodine. The data are taken from Klein's Tables X and XI.

Course of hydrolysis			Effect of enzyme concentration	
Time	Extent of cleavage	$k = \frac{1}{t} \times \log \frac{a}{a - x}$	Relative enzyme concentration	Cleavage in 1 hr.
min.	*per cent*			*per cent*
0		0.020	1.0	63
3	13	0.017	0.5	50
7	25	0.014	0.25	41
15	38	0.010	0.125	30
30	49	0.006	0.0625	18
60	57	0.004		
120	67	0.002		
480	86	0.001		

Use of Reciprocal of the Time Required to Effect a Given Change As a Measure of Reaction Velocity—In 1915 Arrhenius (18) formulated empirically, on the basis of data collected by previous workers, the *Qt* rule: that the product of the time required for an enzyme to effect a given change in a substrate and the concentration of the enzyme was constant. In 1918 Osterhout (4) noted, as has already been mentioned in this paper, that in monomolecular, higher order, consecutive, or autocatalytic reactions, the ratio of the reaction constants, as a condition is varied, is equal to the ratio of the reciprocals of the time required to effect a given change. In the present paper this relationship has been demonstrated generally.

However, the practical situation present in comparing the reaction velocities of most biochemical reactions at different variants of a given condition is that the form of the time-change curve is not known. There must therefore be assurance that the form of the time-action function, whatever it is, does not change. The assurance of such constancy is given by the finding that the ratio of the reciprocals of the time required to effect a given change has the same value for any change in the course of the time-change function.

TABLE IV

Effect of Stage of Reaction on Value of Ratio of Reaction Time at 75 Per Cent by Volume Concentration of Phosphatase Extract to That at 12.5 Per Cent Concentration

Temperature, 25°; concentration of substrate, sodium β-glycerophosphate, 0.0127 M; optimal pH, 9.1; cattle bone phosphatase, Preparation CBH-1-d, used.

P liberated as phosphate per cc. hydrolysis mixture	Time necessary for liberation of phosphate at		Ratio of reciprocals of reaction times
	12.5 per cent concentration of phosphatase	75 per cent concentration of phosphatase	
In absence of added accelerants			
mg.	*min.*	*min.*	
0.02	145	15.2	9.5
0.03	268	23.1	11.5
0.04	440	31.2	14.1
0.05	642	39.6	16.3
0.06	860	48.5	17.7
0.07	1135	57.6	19.7
In presence of 0.00625 M glycine and 0.009 M magnesium			
0.02	40	6.8	5.9
0.03	60	10.1	5.9
0.04	81	13.6	6.0
0.05	102	17.0	6.0
0.06	121	20.2	6.0
0.07	140	23.5	6.0

In Table IV are shown the times necessary to reach certain stages in a typical bone phosphatase hydrolysis of sodium β-glycerophosphate at two different concentrations of bone phosphatase extract: 12.5 per cent and 75 per cent by volume of the hydrolysis mixture. The ratio of these concentrations of enzyme is 6.0. If the reciprocal of the times necessary to liberate 0.02 mg. of phosphorus as inorganic phosphate per cc. of hydrolysis mixture is taken as the measure of reaction velocity, the conclusion is that the enzyme is 9.5 times as active at the higher concentration as at the lower. If the reciprocal of the times necessary for the liberation of 0.04 mg. is used as a measure, the conclusion similarly must be that the enzyme is 14.1 times as active, and at 0.07 mg. the ratio of the reciprocals of the times is 19.7. The arbitrariness of these ratios lies in the fact that the form of the hydrolysis curve is different at the two concentrations of enzyme extract and that, therefore, according to the derivation of the relationships between the various measures of reaction velocity, the reciprocals of the times necessary to effect a given change cannot in this case be properly used as a measure of reaction velocity.

The form of the time-action function is different at the two concentrations of enzyme extract because, as is evident from previous work (1) the concentration of α-amino acids (present as products of autolysis) is greater at the higher concentration of enzyme extract and hence prevents, to a larger extent, the inactivation of the phosphatase during its action on the substrate. Table IV shows that at a concentration of glycine, 0.00625 M, optimal in the prevention of such inactivation, and a concentration of magnesium ion, 0.009 M, optimal in increasing the velocity with which the reaction starts, the ratio of the reciprocal of the time necessary to effect a given change at a 75 per cent concentration of phosphatase extract to the reciprocal of the time necessary to effect the same change at 12.5 per cent concentration is the same, 6.0, at different stages of the reaction. This constitutes assurance that the form of the time-action curve remains constant under these conditions and that, therefore, the reciprocal of the time may be correctly used as a measure of reaction velocity.[4]

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[4] When the form of a function alters as a given condition is varied, the ratios of the reciprocals of the time required to effect a given change cannot be used to represent the ratios of the actual rates of change, even under the given different conditions. This may be illustrated with known functions. *Assume*, for instance, that a reaction proceeding according to the monomolecular expression, $k_m = (1/t) \ln (a/(a - x))$, where $k_m = 0.0100$, at one

Examination of data in the literature shows that a change in the form of the time-action function, as the enzyme concentration is varied, is not unique for phosphatase but occurs for other enzyme preparations. Thus in Sjöqvist's (19) data on the digestion of albumin by pepsin, 4-fold the concentration of pepsin shows the reciprocal of the time to be 3.5 times as large when the reaction is 20 per cent complete, 4.2 times as large at 30 per cent change, and 4.8 at 40 per cent. According to the data of Morgulis (20) on catalase, a 2-fold concentration of catalase shows the ratio of the reciprocals of the times to be 1.9 when 25 cc. of oxygen are evolved, 2.25 when 50 cc. are evolved, and 3.7 when 75 cc. are given off. On the other hand, the data of Nelson and Hitchcock (13) on invertase and of Van Slyke and Cullen (8) on urease show that, for the preparations used, the ratio of the reciprocals of the time necessary to reach a given stage for different concentrations of enzyme is constant at different stages of the reaction. The form of the time-action curve does not change with variation in enzyme concentration.

Use of Reaction Velocity Measures in Formulation of General Kinetic Relationships—It is of importance to determine to what extent general formulations of the relations between the rate of reaction and other factors such as pH, temperature, enzyme concentration, etc., have been based on the correct use of different measures of reaction velocity. In illustration of this problem, there will be discussed briefly the relations between (*a*) reaction velocity and enzyme concentration and (*b*) reaction velocity and temperature. It is hoped to consider the latter relation in greater detail in a subsequent paper.

With regard to the relation between enzyme concentration and

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temperature, proceeds according to the bimolecular expression, $k_b = (1/t) \times (x/a(a - x))$, where $k_b = 0.000100$ at another temperature. The instantaneous rates of change, dx/dt, at various points may be obtained from the derivatives of these expressions, and the reciprocals of the times required to effect a given change by direct substitution in the integral expressions. The ratio of the instantaneous rate of change in the monomolecular course to that in the bimolecular is, at a point corresponding to a 10 per cent change, 1.11, whereas the ratio of the reciprocals of the times is 1.05. At a 30 per cent change, the ratio of the instantaneous rates of change is 1.43, of the reciprocals of the times, 1.20; at 70 per cent change, the ratios are, respectively, 3.33 and 1.94.

reaction velocity, Haldane (21) writes, "The most notable real exceptions [that is, to the law that the velocity of the reaction is directly proportional to the enzyme concentration] are to be found among the proteases. Many authors have found that the velocity of digestion was roughly proportional to the square root of the concentration of pepsin." Waksman and Davison (22) state, "With certain other enzymes, however, the velocity is proportional to the square root of the concentrations—the so-called Schütz and Borissov law."

As the writer has pointed out (23), this formulation is founded on an incorrect use of a measure of reaction velocity. The data upon which the above statements are usually based are those of Schütz (24). Reference to his original data reveals that in the presence of varying concentrations of pepsin, the amounts of albumin digested at the end of a given time, 16 hours, were proportional to the square root of the enzyme concentration. Later work, notably that of Arrhenius (25), showed that at a given concentration of pepsin, the amount of protein digestion could be fairly well expressed as being proportional to the square root of the time, for about the first 50 per cent of the hydrolysis.

At two concentrations of pepsin, A and B, the course of the action may then be expressed as follows: $k_A = (1/t) \times x^2$ and $k_B = (1/t) \times x^2$, where x is the amount of protein digested in time, t. For a given value of t as, for example, 16 hours in Schütz' experiments, the ratio of the reaction constants is $k_A/k_B = x_A{}^2/x_B{}^2$; that is, as the enzyme concentration is varied, the ratios of the reaction constants, which are a proper measure of the velocity of the reaction, are equal not to the ratios of the amounts changed in a given time, but to the ratios of the squares of these amounts. According to the data of Schütz, the amounts changed in a given time are proportional to the square root of the enzyme concentrations, or the squares of these amounts are directly proportional to the enzyme concentrations: $x_A{}^2/x_B{}^2 = E_A/E_B$. Since, as shown above, $x_A{}^2/x_B{}^2 = k_A/k_B$, then $k_A/k_B = E_A/E_B$, or the reaction velocity is directly proportional to the concentration of enzyme, not to the square root thereof. Schütz' own data therefore contribute not to a separate rule but to the rule, established so widely for many enzymes, of direct proportionality between enzyme concentration and reaction velocity.

The Arrhenius expression for the relation between temperature and reaction velocity is, in its differential form, $(d\ln k)/dT = E/RT^2$, where k is the reaction velocity expressed in terms of the reaction constant, R is the gas constant, T is the absolute temperature, and E is a constant known as the critical increment. The value of E is obtained by plotting $\ln k$ against $1/T$ and multiplying the slope of the theoretically straight line by R.

The Arrhenius equation has been found to hold, that is E is constant as T is varied, for a great many chemical reactions. However, with regard to enzymic reactions, the opinion seems to be general (22, 26) that the Arrhenius equation does not hold in most instances. Thus Moelwyn-Hughes (27) states, "Of the vast number of reactions occurring in solution only a very small number fail to conform with the Arrhenius equation. *Enzymic reactions are a notable exception.*"[5]

Though it is not desired to decide here the question of the general applicability of the Arrhenius expression to enzymic reactions, it may be shown that data, brought forward as basis for the above statement that enzymic reactions do not conform with the Arrhenius equation, offer questionable support, since the measures of reaction velocity employed are usually incorrect. Thus, for the hydrolysis of ethyl butyrate by lipase, Moelwyn-Hughes (28) calculates that E, the critical increment, decreases as the temperature rises. Its value is 6200 calories per gm. molecule for 0–10°, 4810 calories for 10–20°, and 4060 calories for the range between 20–30°. But reference to the original data, that of Kastle and Loevenhart (29), upon which these calculations are based, reveals that the measure of reaction velocity employed is the amount hydrolyzed in 30 minutes.

It has been shown, in the course of the present paper, that the concentration of reactant changed in a given time at the beginning of the reaction may properly be used as a measure of reaction velocity (that is, in place of the reaction constant) when the course of the reaction during that stage is of 0 order. There is no assurance, in the data of Kastle and Loevenhart, that this is the case. On the contrary, the only data (29, 30) given for the course of the reaction show that the rate of reaction is of much higher order than 0; values calculated as monomolecular constants fall very rapidly during the period of 30 minutes.

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[5] The italics are mine.

On the other hand, Moelwyn-Hughes notes, as somewhat of an exception, that the critical increment determined by Nelson and Bloomfield (31) for the hydrolysis of sucrose by yeast invertase at optimal pH 4.5 remains constant as the temperature varies, being about 8700 calories. But reference to the original data here shows that a reaction constant, *n*, has properly been employed as the measure of reaction velocity. This reaction constant was so defined empirically (13) that it was a precise formulation of the relation between the concentration of sucrose present and the instantaneous rate of change throughout the entire course of the reaction, and was found to hold for variation in pH, temperature, and enzyme concentration.

The preceding examples illustrate most forcibly, then, the possibility that in many biochemical reactions the velocity of reaction may be incorrectly expressed by various measures, that the use of such measures may lead to unjustified or even incorrect generalizations, and that it is necessary, in utilizing the data of previous investigators on the velocity of various biochemical reactions, to reconsider the nature of the measures they employed.

SUMMARY

1. An investigation has been made into the question of the expression of the velocity of biochemical reactions. The formulation of the time course is discussed in terms of mass law assumptions. The use of the values, calculated as supposed reaction constants from such formulations, as measures of reaction velocity, is considered in various types of reactions.

2. It is shown for those reactions the time course of which cannot be expressed mathematically that, as a given condition is changed, the ratio of the reaction constants, though unknown, is equal to the ratio of the reciprocals of the times required to effect a given change, and also to the *functions* of the amounts changed in a given time, provided that the form of the function remains constant.

3. Since, in reactions of 0 order, the ratio of the functions of the amounts changed in a given time is, in fact, the ratio of the amounts themselves, the latter may correctly be used as a measure

of the reaction velocity for that initial portion of the time course which is actually found to be of 0 order.

4. In order to use the reciprocal of the time necessary to effect a given change correctly as a measure of reaction velocity, there must be assurance that the form of the function does not change with variation in condition. Such assurance is given by the finding that the ratios of the reciprocals of the time yield the same value at different points in the course of the time-change curve.

5. The above principles are illustrated by reference to the phosphatase hydrolysis of sodium β-glycerophosphate and to enzymic reactions studied by previous investigators.

6. In illustration of the necessity for scrutinizing generalizations based on data employing the different measures of reaction velocity, the Schütz-Borissov law for the relation between enzyme concentration and reaction velocity, and the application of the Arrhenius equation to the relation between temperature and velocity of enzymic reactions are examined briefly.

BIBLIOGRAPHY

1. Bodansky, O., *J. Biol. Chem.*, **114**, 273 (1936); **115**, 101 (1936).
2. Bodansky, O., *J. Biol. Chem.*, **118**, 341 (1937).
3. Reed, L. J., and Theriault, E. J., *J. Physic. Chem.*, **35**, 673, 950 (1931).
4. Osterhout, W. J. V., *Science*, **48**, 172 (1918).
5. Rosanoff, M. A., Clark, R. H., and Sibley, R. L., *J. Am. Chem. Soc.*, **33**, 1911 (1911).
6. Pennycuick, S. W., *J. Am. Chem. Soc.*, **48**, 6 (1926).
7. Nelson, J. M., *Chem. Rev.*, **12**, 1 (1933).
8. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19**, 141 (1914).
9. Northrop, J., *J. Gen. Physiol.*, **6**, 429 (1924).
10. Barron, E. S. G., *J. Biol. Chem.*, **113**, 695 (1936).
11. Willstätter, R., Graser, J., and Kuhn, R., *Z. physiol. Chem.*, **123**, 1 (1922).
12. Vosburgh, W. C., *J. Am. Chem. Soc.*, **43**, 219 (1921).
13. Nelson, J. M., and Hitchcock, D., *J. Am. Chem. Soc.*, **43**, 2632 (1921).
14. Nelson, J. M., and Hollander, F., *J. Biol. Chem.*, **58**, 291 (1923–24).
15. Bodansky, O., unpublished data.
16. Olsson, W., *Z. physiol. Chem.*, **126**, 29 (1923).
17. Klein, W., *Z. physiol. Chem.*, **231**, 125 (1935).
18. Arrhenius, S., Quantitative laws in biological chemistry, London, 45 (1915).
19. Sjöqvist, J., *Skand. Arch. Physiol.*, **5**, 317 (1895).
20. Morgulis, S., *J. Biol. Chem.*, **47**, 341 (1921).

21. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London and New York, 11 (1930).
22. Waksman, S., and Davison, W. C., Enzymes, Baltimore, 38, 44 (1926).
23. Bodansky, O., *Science*, **86**, 52 (1937).
24. Schütz, E., *Z. physiol. Chem.*, **9**, 577 (1885).
25. Arrhenius, S., *Medd. Vetenskapsakad. Nobelinst.*, **1**, 1 (1908).
26. Oppenheimer, C., Lehrbuch der Enzyme, Leipsic, 97 (1927).
27. Moelwyn-Hughes, E. A., The kinetics of reactions in solution, Oxford, 50 (1933).
28. Moelwyn-Hughes, E. A., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipsic, **2**, 1 (1933).
29. Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, **24**, 491 (1900).
30. Kastle, J. H., Johnston, M. E., and Elvove, E., *Am. Chem. J.*, **31**, 521 (1904).
31. Nelson, J. M., and Bloomfield, G., *J. Am. Chem. Soc.*, **46**, 1025 (1924).

ON A CATALYTICALLY INDUCED REACTION RESEMBLING THE CANNIZZARO REACTION

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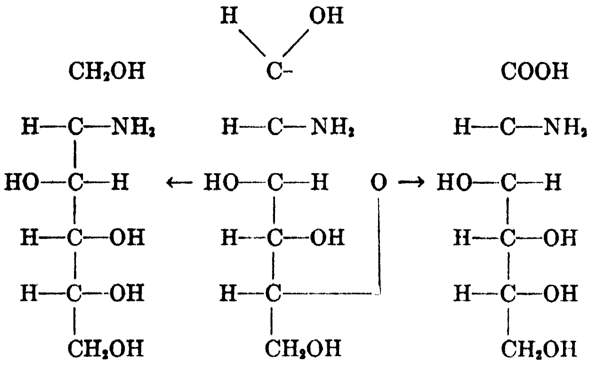
When glucosamine is agitated in an atmosphere of hydrogen in the presence of a platinum catalyst, only 50 per cent of the sugar is reduced to aminosorbitol and the remaining 50 per cent is oxidized to glucosaminic acid. The reaction proceeds in the following way.



II I III

This reaction resembles the well known Cannizzaro reaction which consists of an oxidation-reduction process leading from an aldehyde to equimolecular proportions of acid and carbinol. The Cannizzaro reaction is generally regarded as a homogeneous reaction catalyzed by hydroxyl ions. It is common in the field of aromatic chemistry, where it serves for preparative purposes, but it is not so common in the field of aliphatic aldehydes. In animal tissues and in plants there are present enzymes which have equal power to effect a Cannizzaro reaction in the aliphatic as well as in the aromatic aldehydes. The reaction plays a prominent part in the process of fermentation.

The resemblance of the reaction induced by a platinum catalyst to that induced by the biocatalysts called for a more detailed study of its mechanism.

Subsequently it was found that the products of the reaction produced by contact with platinum catalysts varied with changes in the external conditions, the hydrion concentration and the hydrogen gas pressure being the main factors influencing the course of the reaction. It should be mentioned that the mode of preparation of the catalyst has apparently no effect on the outcome of the reaction. Thus, Adams' catalyst—whether reduced or not prior to use—as well as platinum black prepared according to the directions of Willstätter, acted in a similar way. It also should be mentioned that on contact with the catalyst in the absence of hydrogen, glucosamine remained unchanged. Likewise, a reaction did not take place in an atmosphere of hydrogen in the absence of the catalyst. The latter fact shows that the hydrion concentration due to glucosamine of itself is not capable of catalyzing a Cannizzaro reaction.

Methods of Analysis—The quantity of acid formed was determined by titration to pH 4.5 and, inasmuch as volatile acids could not be detected in the reaction products, the increase of acidity was attributed to the formation of glucosaminic acid. The amount of isolated glucosaminic acid agreed as well as could be expected with that calculated by titration.

The loss in glucosamine was determined by titration according to Hanes-Hagedorn and Jensen.[1]

In all cases an aqueous solution of the substance and the catalyst were placed in the Adams' apparatus in an atmosphere of hydrogen. In all experiments save a few the pressure of the gas was 35 pounds per sq. inch; in the exceptional cases the pressure was either atmospheric or less.

The results of the experiments are given in Table I. From this table it can be seen that: (1) Free glucosamine yields approximately equimolecular proportions of glucosaminic acid and aminosorbitol. There seems to be a slightly higher production of aminosorbitol When the hydrogen pressure was reduced to atmospheric, an excess of acid as compared with aminosorbitol was formed. (2) When glucosamine hydrochloride, or N-acetylglucosamine is subjected to the same treatment as in (1), then only one product is formed, namely aminosorbitol, or its acetate, the reduction of the N-acetylglucosamine proceeding at a considerably slower rate than that of the hydrochloride. (3) When the gas pressure in the apparatus is reduced below atmospheric pressure, the catalyst having been reduced prior to the introduction of the glucosamine solution—the solution containing, in addition to glucosamine, sodium hydroxide at 0.05 N concentration—then the product of the reaction is only glucosaminic acid.

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[1] Hanes, C., *Biochem. J.*, **23**, 99 (1929).

TABLE I

Proportions of Acids and Polyhydric Amino Alcohols Formed from Glucosamine under Different External Conditions on Contact with Platinum Catalyst in an Atmosphere of Hydrogen

	Pressure per sq. in.	Glucosamine	Amino alcohol	Amino acid
		per cent	*per cent*	*per cent*
Glucosamine hydrochloride + Adams' catalyst + hydrogen gas	35 pounds	0	100	0
N-Acetylglucosamine + Adams' catalyst + hydrogen gas	35 "	0	100	0
Glucosamine + Adams' catalyst + hydrogen gas	35 "	0	53	47
Glucosamine + Adams' catalyst reduced + hydrogen gas	35 "	0	53	47
Glucosamine + Adams' catalyst + hydrogen gas + NaOH, 0.05 N	35 "	0	48	52
Glucosamine + Adams' catalyst reduced + hydrogen gas	Much reduced	16	3	81
Glucosamine + Adams' catalyst reduced + hydrogen gas + NaOH, 0.05 N	" "	0	0	100
Glucosamine + Adams' catalyst + hydrogen gas, *t* 8°, time 72 hrs.	Atmospheric	0	46	54
Glucosamine + Adams' catalyst + hydrogen gas, *t* 67°, time 1 hr.	"	0	44	56

Kinetics of Oxidation-Reduction Reaction—The progress of the reaction of free glucosamine in an atmosphere of hydrogen in contact with Adams' catalyst under 35 pounds pressure is given in

Table II. The progress of the reaction was measured by titrating the amount of acid formed (x), the amount of glucosamine remaining at time t being ($a - 2x$).

TABLE II

Rates of Formation of Glucosamine Acid from Glucosamine on Contact with Platinum Catalyst in an Atmosphere of Hydrogen

Time	Volume of 0.020 N HCl to neutralise base present	Volume of 0.020 N acid formed during reaction x	$k = \frac{1}{t}\log\frac{a}{a-2x}$
hrs.	*cc.*	*cc.*	
0	6.65 (a)	0.00	
1	5.21	0.72	0.1059
2	3.99	1.33	0.1109
3	4.05	1.80	0.1129
4	2.35	2.15	0.1129
5	1.85	2.40	0.1111
6	1.57	2.54	0.1046
7	1.41	2.62	0.0962
12	0.81	2.92	0.0762
24	0.41	3.02	0.0432
48	0.35	3.10	0.0244

TABLE III

Rate of Disappearance of Glucosamine

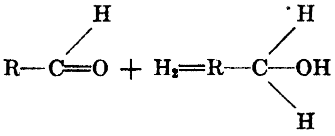
Time	0.01 N $Na_2S_2O_3 \Leftrightarrow$ sugar present	Sugar present, $a - x$	a	$k = \frac{1}{t}\log\frac{a}{a-x}$
hrs.	*ml.*	*mg.*		
0	9.09	3.05		
1	6.56	2.23	2.23	
2	5.32	1.81		0.0930
3	4.25	1.45		0.0934
4	2.75	0.95		0.0924
6	2.04	0.70		0.0838
10	1.36	0.47		0.0676

From Table II it may be seen that the reaction followed a monomolecular course until the reaction was over 80 per cent completed. In these experiments no special precautions were used to maintain constant temperature or constant pH. In a second experiment, the reaction was carried out at constant pH and constant temperature. However, the hydrogen pressure was reduced to 1 atmosphere. In this case the reaction seemed to follow a monomolecular course until about 70 per cent of the sugar was changed into glucosaminic acid and into aminosorbitol. The constancy of k_1 (Table III), however, is spurious for there was observed in every interval a noticeable higher production of acid over that of alcohol.

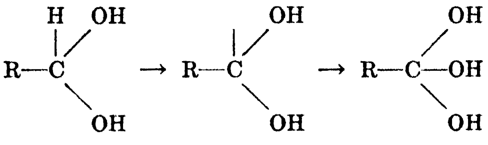
Effect of Temperature—Most of the experiments were performed at room temperature at a pressure of 35 pounds per sq. inch. For the study of the temperature effect an apparatus was constructed consisting of the conventional glass vessel (Ente) used for hydrogenation experiments, provided with a jacket for circulating water. The rate of reaction was measured at 8° and at 67°. In each case 56 per cent of glucosaminic acid and 44 per cent of the aminosorbitol were formed at the end of the experiment. This yield was obtained after 72 hours at the lower temperature and after 1 hour at the higher temperature. Hence the reaction approximately doubled for every increment of the temperature of 10°.

Discussion of Results

Inasmuch as the oxidation-reduction reaction of glucosamine does not take place in the absence of the catalyst, the reaction is one of heterogeneous catalysis. At the point of neutrality glucosamine functions as the acceptor of hydrogen and the catalyst as the donor. Thus it is possible that in the reaction of reduction the carbonyl group functions as any unsaturated group.

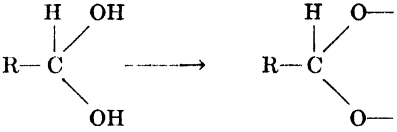


On the other hand, the reaction of oxidation requires the presence of hydroxyl groups. It is therefore possible that this reaction is preceded by the formation of the hydrated form of glucosamine (I)



I II III

which is dehydrogenated by the catalyst into (II), which, in its turn, accepts a hydroxyl group to form the acid. Thus, in the reaction of oxidation the catalyst acts as an acceptor of hydrogen. Whether this is the true mechanism or whether 2 hydrogen atoms are removed synchronously from the hydrated form (I),



III IV

as postulated by Wieland,[2] remains uncertain.

The combined oxidation-reduction reaction takes place when conditions are given for both reactions; namely, sufficient pressure of hydrogen gas to maintain a sufficient supply of platinum dihydride and a sufficient hydroxyl ion concentration to give rise to the hydrogenated form. It would then follow that each reaction takes place on a single molecule of the aldehyde and not on a bimolecular condensation product. Thus the reactions of reduction and oxidation are two separate reactions. The latter is dependent upon the first only to the extent as decrease in the mass of the platinum dihydride enhances the process of oxidation.

It may be of interest now to consider the extent to which the platinum catalyst may serve as a model of the enzymatic Cannizzaro reaction and perhaps even of the homogeneous Cannizzaro reaction.

Before we enter into this analysis, it should be pointed out that reactions similar to that described above have been observed with substances other than glucosamine, although, to our knowledge, the simultaneous oxidation and reduction have never been observed under the conditions described above. Simple monoses were reduced catalytically to corresponding polyhydric alcohols in this laboratory[3] and later by Glattfeld.[4] Oxidation of glucose to carbon dioxide was accomplished by Wieland with large excess of catalyst (2.7 gm. for 0.125 gm. of sugar) under a pressure of hydrogen much below atmospheric pressure.

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[2] Wieland, H., *Ber. chem. Ges.*, **46**, 3327 (1913).

[3] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **98**, 9 (1932).

[4] Glattfeld, J. W. E., and Schimpff, G. W., *J. Am. Chem. Soc.*, **57**, 2204 (1935).

A reaction resembling the Cannizzaro, that is equimolecular production of polyhydric alcohol and corresponding carboxylic acid from simple monoses, has been recently reported by Bach *et al.*[5] in 0.5 N solution of alkali and under a pressure of hydrogen much below atmospheric. Under such conditions glucosamine yields only glucosaminic acid. In the experiments by Bach *et al.* the *concentration of the alkali was about 10 times as high as in the experiments with glucosamine.* It is possible that the differences in the outcome of the reaction might be attributed to this cause.[6]

It may be mentioned here that simultaneous hydrogenation and dehydrogenation of unsaturated cyclic hydrocarbons was observed by Zeiling.[7] The reaction, however, took place at a higher temperature and in an atmosphere of carbon dioxide, thus again under conditions totally different from those of our experiment.

Comparison of Reactions Induced by Chemical Catalyst and by Biocatalyst—As far as it is known, the enzyme, though not isolated in pure form, is definitely a substance of high molecular weight with colloidal properties. Hence the reaction by contact with the enzyme may be regarded as a heterogeneous reaction. Similarly to the platinum catalyst, the biocatalyst is capable of producing either oxidation or reduction, as well as oxidation and reduction simultaneously. The hydrion concentration is an important factor influencing the rate and the outcome of the enzymatic reaction as it does in the reaction on the platinum catalyst.[8] Thus there is a possibility that a more detailed study of the enzymatic reaction may reveal greater similarity in the action of the two catalysts.

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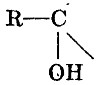
[5] Bach, A., Alekseeva, E. P., and Dreving, V. P., *Biochimia,* **1,** 75 (1936).

[6] The publication of Bach *et al.* in a new Russian journal reached us very recently, long after our observations were made. Our own observations were made in course of renewed efforts to determine the configuration of glucosamine. It was intended to reduce aminosorbitol to 2-aminohexane whose configuration has been correlated by Levene and Mardasheff to α-aminocaproic acid. Great difficulties, however, are being encountered in this work which accounts for the delay in publishing the present article.

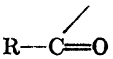
[7] Zeiling, A., *Ber. chem. Ges.*, **57,** 2055, 2058 (1924).

[8] Batelli, F., and Stern, L., *Biochem. Z.*, **28,** 159 (1910). Parnas, J., *Biochem. Z.*, **28,** 274 (1910). Quastel, S. H., and Wheatley, A. H. M., *Biochem. J.*, **26,** 2167 (1932). Batelli, F., and Stern, L., *Biochem. Z.*, **29,** 130 (1910).

Homogeneous and Heterogeneous Cannizzaro Reactions—Although the majority of workers accept complex formation as the first step of the homogeneous reaction of Cannizzaro,[9] yet there are authors accepting dehydrogenation as the first step of the reaction with the formation either of a divalent carbon compound[10]



or of a free radicle[11]



Furthermore, it has been observed by Varley[12] that the reaction of Cannizzaro may be shifted by external conditions towards production of the carbinol in excess of the acid. Recently Kuhn and Meyer[13] produced evidence to show that in a medium free from contamination ("sterile") autoxidation of benzaldehyde does not take place. Thus there is not excluded the possibility that a catalyst other than the hydroxyl ion induces the so called homogeneous Cannizzaro reaction.

EXPERIMENTAL

Reduction of Free Glucosamine—10 gm. of free glucosamine were dissolved in 200 cc. of water (distilled from glass vessels) and to this solution 1 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen, at a pressure of 35 pounds per sq. inch, for 2 days, at the end of which time the solution failed to reduce boiling Fehling's solution.

The catalyst was removed by filtration, washed well with water, and then kept in the refrigerator for further reductions. The filtrate and washings were combined and concentrated under reduced pressure at 40° to about 25 to 30 cc.

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[9] Tischtschenko, W., *J. Russ. Physic.-Chem. Soc.*, **38**, 482 (1937). Claisen, L., *Ber. chem. Ges.*, **20**, 646 (1887). Kohn, C. A., and Trantom, W. J., *J. Chem. Soc.*, **75**, 1155 (1899). Lachman, A., *J. Am. Chem. Soc.*, **45**, 2358 (1923). Merwein, H., and Schmidt, R., *Ann. Chem.*, **444**, 221 (1925).

[10] Nef, J. V., *Ann. Chem.*, **298**, 301 (1897).

[11] Haber, F., and Willstätter, R., *Ber. chem. Ges.*, **64**, 2844 (1931).

[12] Varley, A., *Bull. Soc. chim.*, **37**, 537 (1925).

[13] Kuhn, R., and Meyer, K., *Naturwissenschaften*, **16**, 1028 (1928).

The experiment from this point is divided into two parts; namely, *A. Isolation of glucosaminic acid* and *B. Preparation of crystalline 2-aminosorbitol hexaacetate.*

A. Isolation of Glucosaminic Acid

Absolute ethyl alcohol was then added to the above concentrated solution until crystallization was complete. After the material stood in the refrigerator for several hours, the crystals were removed by filtration and washed first with cold 85 per cent alcohol and then with absolute alcohol. The mother liquor and washings were concentrated under reduced pressure to about 20 cc. and from this concentrate another crop of crystals was obtained. In all, four crops of crystals were obtained in this way. Yield, 4.0 gm. (The filtrates and washings were concentrated to a thick sirup and kept in the refrigerator. The isolation of a product from this material will be discussed in Part B.)

The product was recrystallized five times by dissolving in a small amount of water and then adding absolute ethyl alcohol. This gave a pure material having the following specific rotation

$$[\alpha]_D^{20} = \frac{-1.10^\circ \times 100}{1 \times 8.24} = -13.4^\circ \text{ (in 20\% hydrochloric acid)}$$

The substance had the following composition.

5.412 mg. substance: 7.305 mg. CO_2 and 3.170 mg. H_2O
5.400 " " : 0.352 cc. N_2 (760 mm. at 25°)
$C_6H_{13}O_6N$. Calculated. C 36.91, H 6.8, N 7.6
Found. " 36.75, " 6.5, " 7.5

B. Preparation of Crystalline 2-Aminosorbitol Hexaacetate

The sirupy residue remaining after the removal of the glucosaminic acid was dried to constant weight by concentrating under reduced pressure with small portions of benzene. The sirup was acetylated by warming and triturating for 1 hour with a mixture of 30 cc. of acetic anhydride and 30 cc. of dry pyridine. At the end of this time the sirup had gone into solution, the solution having a brown color. The mixture was kept at room temperature overnight and then poured into ice and water.

The water solution was now extracted with three portions of chloroform and the chloroform extract washed successively with water, 5 per cent sulfuric acid, sodium bicarbonate solution, and finally with water. It was dried with anhydrous sodium sulfate and concentrated under reduced pressure, at 40°, to a thick sirup. All attempts to crystallize this sirup failed.

The dried sirup was therefore distilled under a high vacuum and a yellow viscous sirup was collected distilling between 160–180° (bath temperature 200–210°) and 0.3 mm. Yield, 8.0 gm. This sirup started to crystallize after standing at room temperature in an open test-tube for several weeks. When the main portion of the sirup was dissolved in a small volume of absolute ethyl alcohol, and an equal volume of ether and of pentane added until the solution became opalescent, followed by nucleation with the crystals, the substance settled out in crystalline form. After two more recrystallizations, the substance was analytically pure. Yield, 4.5 gm. The substance melted at 99–100° and had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.83^\circ \times 100}{2 \times 4.36} = +21.0^\circ \text{ (in chloroform)}$$

The substance is soluble in ethyl alcohol, methyl alcohol, acetone, water, benzene, chloroform, and ethyl acetate. It is moderately soluble in ether and is practically insoluble in pentane.

The substance had the following composition.

4.652 mg. substance: 8.510 mg. CO_2 and 2.580 mg. H_2O
9.198 " " : 0.263 cc. N_2 (771 mm. at 25°)
7.271 " " : 9.843 " 0.01 N $Na_2S_2O_3$[14]
$C_{18}H_{27}O_{11}N$. Calculated. C 49.90, H 6.3, N 3.2, $COCH_3$ 58.2
Found. " 49.89, " 6.2, " 3.3, " 58.2

Preparation of N-Acetyl-2-Aminosorbitol—15 gm. of pure 2-aminosorbitol hexaacetate were dissolved in 250 cc. of absolute methyl alcohol and the solution cooled in an ice-alcohol mixture. 10 cc. of 0.5 N barium methylate solution in absolute methyl alcohol[15] were then added and the mixture kept in the refrigerator for

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[14] Elek, A., and Harte, R. A., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 267 (1936).

[15] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 631 (1931).

2 days during which time a crystalline deposit formed. This was filtered off and washed with cold absolute methyl alcohol. Yield, 2.0 gm.

The filtrate and washings were diluted with 120 cc. of water and the solution was saturated with carbon dioxide. The solution was decolorized by boiling with charcoal. The filtrate was concentrated under reduced pressure at 40° and the residue recrystallized from absolute ethyl alcohol giving another crop of crystals weighing 4.5 gm. Total yield, 6.5 gm.

The substance was pure after three recrystallizations from absolute ethyl alcohol and had a melting point of 152–153°. Its specific rotation was

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-0.93^\circ \times 100}{2 \times 4.492} = -10.4^\circ \text{ (in water)}$$

The substance is soluble in warm ethyl or methyl alcohol and water. It is practically insoluble in ether, acetone, chloroform, and 30–40° petroleum ether.

The substance had the following composition.

4.912 mg. substance: 7.780 mg. CO_2 and 3.195 mg. H_2O
25.30 " " : 0.00 cc. amino N_2 (757 mm. at 27°)
$C_{18}H_{17}O_6N$. Calculated. C 43.10, H 7.7, amino N 0.00
Found. " 43.19, " 7.82, " " 0.00

Action of Adams' Catalyst on Free Glucosamine in Absence of Hydrogen[16]—1 gm. of free glucosamine was dissolved in 150 cc. of water (distilled from glass vessels) and to this 0.2 gm. of unreduced Adams' catalyst was added. This was shaken in the Adams' shaking machine, in air atmosphere, for 2 days. A 5 cc. portion of the solution at the beginning of the reaction had the same reducing power as a 5 cc. portion at the end of the 2nd day, thus indicating that no reaction had taken place.

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[16] In this and the following experiments the formation of amino acid was determined by titration with 0.02 N hydrochloric acid to pH 4.5, with sodium alizarin sulfonate as an indicator. In the experiments to which alkali was added, the number of ml. of 0.02 N acid required to neutralize this alkali was deducted from the total volume used. At the end of each experiment the concentration of remaining unchanged glucosamine was determined by the Hanes-Hagedorn and Jensen method.[1]

Action of Hydrogen Gas on Free Glucosamine in Absence of Adams' Catalyst—1 gm. of free glucosamine was dissolved in 150 cc. of water (distilled from glass vessels) and then shaken in the Adams' shaking machine with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. The reducing power of equal portions taken at the beginning and at the end of the experiment was identical.

Action of Adams' Catalyst in an Atmosphere of Hydrogen on Glucosamine Hydrochloride—1 gm. of pure glucosamine hydrochloride was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. The acid content and reducing sugar content in aliquot portions of the solution were then estimated.

The mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 3 days, at the end of which time a test portion of the solution failed to reduce boiling Fehling's solution. The titration of aliquot portions of this final solution revealed that no acid was formed during the reaction and that no reducing sugars were present.

The catalyst was removed by filtration and the filtrate concentrated to dryness under diminished pressure at 40°. The sirup was crystallized by dissolving in 2 cc. of water and adding 15 cc. of absolute ethanol. Two crops of crystals were obtained in this way. Yield, 0.65 gm. (theoretical yield, 0.70 gm., since 30 cc. of the above reaction mixture had been used for titrations).

The substance was pure after three recrystallizations from water-alcohol and had a melting point of 157–158° and the following specific rotation.

$$[\alpha]_D^{30} = \frac{-0.21° \times 100}{2 \times 4.4} = -2.4° \text{ (in 20\% hydrochloric acid)}$$

The substance is soluble in water and practically insoluble in the usual organic solvents.

It had the following composition.

5.203 mg. substance: 6.310 mg. CO_2 and 3.310 mg. H_2O
5.500 " " : 0.309 cc. N_2 (760 mm. at 27°)
$C_6H_{16}O_5NCl$. Calculated. C 33.10, H 7.4, N 6.4
Found. " 33.07, " 7.1, " 6.4

Action of Adams' Catalyst in an Atmosphere of Hydrogen on N-Acetylglucosamine—N-Acetylglucosamine (0.80 gm.) was dissolved in 100 cc. of water (distilled from glass vessels) and 200 mg. of Adams' catalyst were added. The acid content and reducing sugar in aliquot portions of the solution were then determined.

The mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. The titration of aliquot portions of this solution indicated the total absence of acid and the presence of about 20 per cent reduced material, thus indicating the absence of any oxidation.

Another 100 mg. of catalyst were then added to the above mixture every 24 hours until, at the end of the 10th day, the reduction was complete, since a portion of the final solution failed to reduce boiling Fehling's solution. Titration of an aliquot portion showed the absence of any acid.

TABLE IV

Experimental Data Showing Acid Formation Titrations

2.5 ml. were taken for each titration.

Experiment No.	0.02 N HCl required for titration of sample		Acid formed = $\frac{\text{ml. initial} - \text{ml. final}}{\text{ml. initial}} \times 100$	Unchanged glucosamine
	Initial	Final		
	ml.	*ml.*	*per cent*	*per cent*
I	6.60	3.50	47	0
II	12.85 − 6.20* = 6.65	9.40 − 6.20* = 3.20	52	0
III	6.63	1.28	81	16
IV	12.65 − 6.20* = 6.45	6.25 − 6.20* = 0.05	100	0
V	6.68	3.05	54	0
VI	6.67	2.95	56	0

* Number of ml. of alkali added.

Action of Reduced Adams' Catalyst in an Atmosphere of Hydrogen on Free Glucosamine—Adams' catalyst (100 mg.) was added to 50 cc. of water (distilled from glass vessels) and then shaken with hydrogen in order to reduce the catalyst completely. 500 mg. of free glucosamine were introduced into the apparatus.

This mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. At the end of that time test portions of the solution failed to reduce boiling Fehling's solution. Titration of aliquot portions indicated the formation of 47 per cent acid (Table IV, Experiment I). The remainder was amino alcohol, since a portion of the final solution did not reduce boiling Fehling's solution. These results are the same as in previous experiments in which the catalyst was not reduced prior to the addition of the glucosamine.

Action of Platinum Black in an Atmosphere of Hydrogen on Free Glucosamine—1 gm. of free glucosamine was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of platinum black were added. The mixture was then shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days at the end of which time a test portion of the solution failed to reduce boiling Fehling's solution.

The catalyst was removed by filtration and the filtrate concentrated to a sirup under reduced pressure at 40°. The sirup was dissolved in a small volume of water and absolute ethanol was added until no further increase in the crystalline deposit was observed. It was then placed in the refrigerator until crystallization was complete. (Total yield, 0.40 gm.) These crystals had a composition corresponding to that for glucosaminic acid. (Calculated, C 36.91, H 6.8; found, C 37.13, H 7.05.) The amino alcohol in the alcoholic filtrates was not isolated, since it had been examined in previous experiments.

Action of Adams' Catalyst in an Atmosphere of Hydrogen on Free Glucosamine in Presence of Alkali—Free glucosamine (0.500 gm.) was added to a solution of 25 ml. of water and 25 ml. of 0.1 N NaOH. Then Adams' catalyst (100 mg.) was introduced into the apparatus and the mixture was shaken with hydrogen at a pressure of 35 pounds per sq. inch for 24 hours.

Titrations taken at the beginning and after the elapse of 24 hours indicated the formation of 52 per cent acid (Table IV, Experiment II). The remainder was amino alcohol (no reduction of Fehling's solution by the final solution).

Action of Reduced Adams' Catalyst on Free Glucosamine under Reduced Pressure—To 100 ml. of water (distilled from glass vessels) 100 mg. of Adams' catalyst were added and the mixture shaken with hydrogen for 20 minutes in order to reduce the catalyst completely. Free glucosamine (1 gm.) was then introduced and the air of the apparatus was replaced by hydrogen gas which was then evacuated.

The mixture was next shaken for 24 hours. Titrations of aliquot portions of this solution at the beginning and after the elapse of 42 hours indicated the formation of 81 per cent acid (Table IV, Experiment III) and only a small amount of amino alcohol, since only 84 per cent of the sugar present was reduced. (A 1 ml. portion showed the presence of 3.03 mg. of sugar at the beginning of the reaction and 0.46 mg. at the end of the reaction.)

Action of Adams' Catalyst on Free Glucosamine and Alkali under Reduced Pressure—To 25 cc. of water, Adams' catalyst (100 mg.) was added and this mixture shaken with hydrogen for 20 minutes. The bottle was then opened and 500 mg. of free glucosamine were added together with 25 ml. of 0.1 N sodium hydroxide solution. Hydrogen was now quickly passed into the apparatus and then the whole apparatus evacuated for 10 minutes. This mixture was next shaken under reduced pressure for 72 hours at the end of which time the solution failed to reduce boiling Fehling's solution.

Titrations of aliquot portions of the solution taken at the beginning and after the elapse of 72 hours indicated the complete transformation of the glucosamine into glucosaminic acid (Table IV, Experiment IV) without any noticeable formation of amino alcohol.

Rate of Formation of Glucosaminic Acid in the Reaction between Free Glucosamine and Adams' Catalyst and Hydrogen at 25°—1 gm. of free glucosamine was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. This mixture was then shaken with hydrogen at a pressure of 35 pounds per sq. inch at 25°.

A portion (2.5 cc.) was removed from the reaction mixture at regular intervals and titrated with 0.020 N hydrochloric acid, with sodium alizarin sulfonate as indicator. The data obtained and the value of k, calculated for a first order reaction, are given in Table II. The amount of acid formed was approximately 47 per cent.

Comparison of Rates of Acid Formation at 8° and 67° in Reaction of Free Glucosamine, Adams' Catalyst, and Hydrogen—Free glucosamine (0.5000 gm.) was dissolved in 50 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. The apparatus was enclosed in a water jacket through which water, at a temperature of 67° in one case and 8° in the other, circulated.

The mixtures were shaken with hydrogen at *atmospheric pressure* until all reducing sugar disappeared. Titrations on aliquot portions of the solutions were taken and the amount of acid formed in a given time tabulated. The amount of acid formed at 8° and at 67° was approximately 56 per cent (Table IV, Experiment V and Experiment VI respectively) and this amount of acid was formed in approximately 1 hour at 67° and in approximately 72 hours at 8°.

Rate of Reaction of Glucosamine in Buffered Solution in Presence of Reduced Adams' Catalyst—50 ml. of water and 100 mg. of Adams' catalyst were shaken with hydrogen for 20 minutes. The catalyst was removed by centrifugation and diluted to 50 ml. with an acetate-veronal buffer solution of pH 8.66. To this mixture there was then added 0.500 gm. of free glucosamine and quickly mixed. A 1 ml. portion was immediately removed for sugar estimation and the remainder of the mixture then shaken with hydrogen at atmospheric pressure, in a jacketed vessel cooled with water at a temperature of 20–21°.

A 1 ml. portion was removed at regular intervals for sugar estimation and these values and the value of k are given below. In the calculation of k the concentration of sugar at the end of the 1st hour was used as the initial concentration (a).

OXIDATION AND HYDROLYSIS OF POLYGALACTURONIDE METHYL ESTER TO LEVO-TARTARIC ACID

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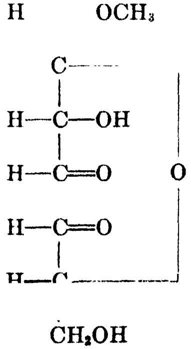
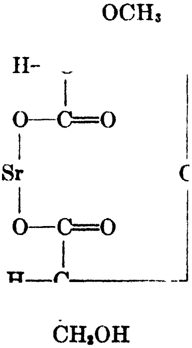
(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 28, 1937)

Malaprade[1] introduced periodic acid for the purpose of dissociating the carbon chain of polyhydric glycols. Criegee,[2] a few years later, introduced lead tetraacetate—which generally acts similarly to periodic acid—as an oxidant, particularly for the purpose of recognizing the presence of two adjacent terminal hydroxyl groups in glycosides. Fleury and Lange,[3] at the same time, recognized that periodic acid acts only in the presence of two adjacent hydroxyl groups. Stimulated by the discrepancies in the results obtained by the action of lead tetraacetate and of periodic acid on sugars, Karrer and Pfaehler[4] investigated the



(*a*)



(*b*)

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[1] Malaprade, L., *Bull. Soc. chim.*, **43**, 683 (1928); **1**, 833 (1934).

[2] Criegee, R., *Ber. chem. Ges.*, **64**, 260 (1931).

[3] Fleury, P., and Lange, J., *Compt. rend. Acad.*, **195**, 1395 (1932); *J. pharm. et chim.*, **17**, 196 (1933).

[4] Karrer, P., and Pfaehler, K., *Helv. chim. acta*, **17**, 766 (1934).

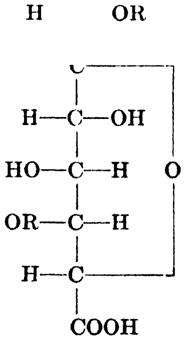
details of the mechanism of the action of periodic acid on methylglucosides and came to the conclusion that formation of formaldehyde during oxidation with periodic acid is preceded by the intermediate formation of an acetal of the structure shown in (*a*).

More recently Jackson and Hudson[5] made a more comprehensive study of the action of periodic acid on glycosides. They oxidized Karrer's dialdehyde into a dicarboxylic acid which was isolated by them in the form of a strontium salt of the composition shown in (*b*).

On further oxidation with subsequent hydrolysis, this product yielded oxalic and glyceric acid. The author offered these findings as direct evidence of the pyranoid structure of the more common glycosides.

Stimulated by the recent applications of periodic acid, particularly those of Jackson and Hudson, we subjected a polygalacturonide prepared by the method of Morell, Baur, and Link[6] to the action of periodic acid. Following the experience of Jackson and Hudson, the oxidation with periodic acid was followed by oxidation with bromine and by hydrolysis. From the product of hydrolysis levo-tartaric acid was isolated in the form of its acid potassium salt.

This finding indicates that the glycosidic units of the polygalacturonate can have only one of the following structures.



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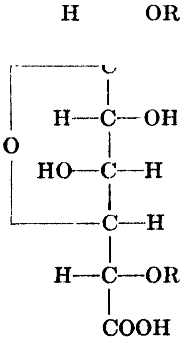
or



II

(R = galacturonide residue)

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[5] Jackson, E. L., and Hudson, C. S., *J. Am. Chem. Soc.*, **58**, 378 (1936); **59**, 994 (1937).

[6] Morell, S., Baur, L., and Link, K. P., *J. Biol. Chem.*, **105**, 1 (1934). Baur, L., and Link, K. P., *J. Biol. Chem.*, **109**, 293 (1935).

Taking the case of pyranoside structure, had R been in position (3), then the substance would not be oxidized by periodic acid; on the other hand, if R was in position (2), the molecule should have been ruptured in two 3-carbon fragments.

In the case of furanoid structure, only when R is in position (5) could the substance be oxidized by periodic acid, for only then does it contain two adjacent unsubstituted hydroxyl groups.

Thus it may be concluded that the hydroxyl groups of carbon atoms (4) and (5) are engaged in the ring closure and in the condensation of the galacturonic acid residues. The special function of each hydroxyl group remains to be established.

EXPERIMENTAL

Oxidation and Hydrolysis of Polygalacturonide Methyl Ester to Levo-Tartaric Acid—Polygalacturonide methyl ester[6] (2.6 gm.) was dissolved in 100 cc. of water. To this was added a solution of 15.8 gm. (6.0 moles) of crystalline periodic acid[7] (H_5IO_6) in 100 cc. of water, and the whole allowed to stand at room temperature overnight, during which time a light orange color developed.

To this solution were added 12 gm. of strontium carbonate in small portions with stirring to neutralize the mineral acids. Carbon dioxide was evolved and the precipitate, consisting of strontium iodate, strontium periodate, and unchanged strontium carbonate, which settled out was removed by filtration. The filtrate was oxidized by adding 5 cc. of bromine and 15 gm. of strontium carbonate with mechanical stirring for 24 hours at room temperature, at the end of which time the excess bromine was removed by aeration. This solution gives no Fehling's reduction unless previously hydrolyzed with acids.

To this mixture 60 cc. of 6 N sulfuric acid (10 to 15 cc. more sulfate ion than required to combine with all the strontium present) was slowly added, and the strontium sulfate removed by filtration. Silver carbonate (about 10 gm.) was now added to the filtrate until a portion tested with saturated silver sulfate solution gave no precipitate. By this procedure were removed all the bromine ions introduced during the bromine oxidation, and the iodate and periodate ions that had escaped precipitation with strontium.

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[7] Manufactured by G. Frederick Smith Chemical Company, Columbus, Ohio.

After the precipitated silver salts were filtered, the filtrate was treated with hydrogen sulfide to free the solution from silver ions, aerated, and filtered. This yielded a colorless filtrate that still contained sufficient sulfuric acid to effect hydrolysis of the glycosidic bonds present in the oxidized polygalacturonide. Accordingly, the solution was maintained at the boiling point under a reflux and the hydrolysis followed polarimetrically. At the end of 13 hours the rotation had become constant and the hydrolysis was judged complete.

The sulfate ions were now removed quantitatively with barium hydroxide, giving a solution that was practically free from all inorganic matter. This was concentrated under diminished pressure to a volume of 5 cc. Potassium hydroxide (6.0 N) was now added until the solution was just alkaline to phenolphthalein. Glacial acetic acid (about 4 cc.) was introduced and crystallization of the acid potassium tartrate was induced by gently rubbing the inner walls of the flask with a glass rod, and was completed by cooling in the refrigerator.

The crystals were filtered off, washed successively with 50 per cent ethanol, 100 per cent ethanol, and ether and dried at 78° over sulfuric acid under diminished pressure. Yield, 0.55 gm.

The product was purified by recrystallizing from 8 cc. of boiling water, cooling, filtering, washing, and drying as before. Its identity as pure levo-potassium acid tartrate was established by its analysis and its optical behavior.

6.521 mg. substance: 3.012 mg. K_2SO_4
$C_4H_5O_6K$ (188.14). Calculated, K 20.78; found, K 20.72

Its specific rotation was

$$[\alpha]_D^{26} = \frac{-0.09^\circ \times 100}{2 \times 0.204} = -22^\circ \text{ (in water)}$$

A single drop of concentrated hydrochloric acid was added to this same solution and after equilibrium was reached the specific rotation had changed to

$$[\alpha]_D^{26} = \frac{-0.06^\circ \times 100}{2 \times 0.20} = -15^\circ \text{ (in water containing traces of } K^+\text{, } H^+\text{, and } Cl^-\text{)}$$

Pribram[8] gives $[\alpha]_D^{20} = +21.57°$ ($c = 0.25$ in water) for the *dextro*-potassium acid tartrate and $[\alpha]_D^{20} = +14.96°$ ($c = 0.20$ in water) for *dextro*-tartaric acid.

We wish to thank the Research Department of the California Fruit Growers Exchange, Ontario, California, for their generous gift of citrus pectin which has served as our source of the polygalacturonide methyl ester.

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[8] Pribram, R., *Monatsh. Chem.*, **14**, 739 (1893).

THE RING STRUCTURE OF α-METHYL-*d*-GALACTURONIDE AND ITS DERIVATIVES

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(Received for publication, June 28, 1937)

A knowledge of the ring structure of methylgalacturonide is essential for the study of the details of the structure of all polygalacturonides. Evidence in favor of the pyranoside structure was given by Morell and Link[1] who compared the rates of hydrolysis of α-methyl-*d*-galacturonide and of α-methyl-*d*-galactopyranoside. Niemann and Link[2] synthesized α-methyl-*d*-galacturonide by oxidation of diacetone-*d*-galactose which was found by Levene and Meyer[3] to have the pyranose structure.

These investigations made the pyranose structure of methyl-*d*-galacturonide very probable. However, direct evidence was much desired.

With this aim in view, methyl-*d*-galacturonic ester prepared by the method of Morell and Link[4] was exhaustively methylated by the method of Purdie. The α-methyl-2,3,4-trimethyl-α-galacturonide methyl ester was obtained in beautifully crystalline form in practically quantitative yield.

From the product of oxidation of the α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester a substance was isolated having the properties of trimethoxy-*l*-araboglutaric acid which was identified as its dimethylamide. This finding established definitely the pyranoside structure of α-methyl-*d*-galacturonide methyl ester and of the many compounds prepared from it, as can be seen from the accompanying formulæ.

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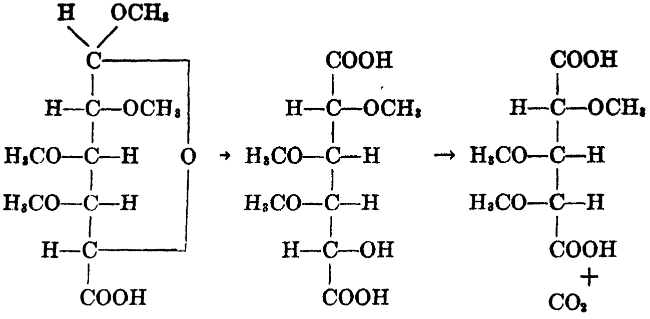
[1] Morell, S., and Link, K. P., *J. Biol. Chem.*, **104**, 183 (1934).

[2] Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 195 (1934).

[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

[4] Morell, S., and Link, K. P., *J. Biol. Chem.*, **100**, 385 (1933).



I II III

Additional evidence in favor of the pyranoside structure of α-methyl-*d*-galacturonide is found in the behavior of its amide with respect to Weerman and to Hofmann reactions. To our knowledge, no attempt had previously been made to degrade uronic amides by one of these methods. Both of these reactions are general for all α-hydroxy acid amides. Hofmann[5] reports in the case of ethoxyacetamide the formation of a mixed urea derivative yielding on hydrolysis formaldehyde. Haworth, Hirst, and Chamberlain[6] report that 2-methylgluconamide does not undergo the Weerman degradation under the usual conditions. This information reached us after our own experiments had been completed. In experiments performed under the conditions given by Weerman,[7] the only product identified by us from α-methyl-*d*-galacturonamide was the starting material in a very considerable quantity. Under the conditions of the Hofmann reaction, the only product isolated was α-methylgalacturonate dihydrate.

Thus, the attempted degradations failed, but the experience brought forth evidence that the hydroxyl group in position (5) of α-methylgalacturonide is engaged in the ring formation; otherwise the degradations should have been successful.

[5] Hofmann, A. W., *Ber. chem. Ges.*, **18**, 2734 (1885).

[6] Haworth, W. N., Hirst, E. L., and Chamberlain, K. A., *J. Chem. Soc.*, 795 (1937).

[7] Weerman, R. A., *Rec. trav. chim. Pays-Bas*, **37**, 16 (1918).

EXPERIMENTAL

Anhydrous-α-Methyl-d-Galacturonide Methyl Ester—α-Methyl-*d*-galacturonide methyl ester monohydrate was prepared by the method of Morell and Link.[4]

The monohydrate was dehydrated by drying under reduced pressure over phosphorus pentoxide for 2 days at 78°. It was then dissolved in boiling absolute ethanol (solubility about 3 gm. in 10 cc.) and slowly cooled to 0°. Beautiful flaky crystals appeared which melted sharply at 148° and whose composition agreed with that of anhydrous α-methyl-*d*-galacturonide methyl ester.

4.206 mg. substance: 6.675 mg. CO_2 and 2.415 mg. H_2O

$C_8H_{14}O_7$.	Calculated.	C 43.22, H 6.4
222.11	Found.	" 43.26, " 6.4

α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—Anhydrous α-methyl-*d*-galacturonide methyl ester (8.0 gm.) was placed in a 3-necked flask fitted with an efficient motor stirrer and a reflux condenser. It was dissolved in 35 cc. of anhydrous methanol at 60° and then 35 cc. of methyl iodide were added through the reflux condenser. Vigorous stirring was commenced and continued throughout the reaction. Silver oxide (40.0 gm.) was added portionwise over a period of 8 hours, and the reaction temperature was maintained at 40°. At the end of the 4th hour an additional 20 cc. of methyl iodide were added and at the end of the 6th hour a further 10 cc. of methyl iodide were introduced. (Portionwise addition of the methyl iodide is necessary because of the insolubility of the unmethylated product in this reagent, while portionwise addition of silver oxide is performed so that fresh, unreacted surfaces of this solid will always be available for methylation.[8])

At the end of the 8th hour the stirring was stopped, the reaction mixture allowed to cool to room temperature, and the whole left to stand overnight. 50 cc. of methanol were now added, and the mixture vigorously stirred for a few minutes and then filtered by suction to separate the silver salts from the solution. The silver salts were then extracted four times with 30 cc. portions of boiling methanol. These washings were combined with the main filtrate and the whole was concentrated under diminished pressure to a thick syrup with a bath temperature of less than 40°.

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[8] Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, **4**, 221 (1931).

This syrup was next remethylated twice more, each time with 35 cc. of methyl iodide at 40°, in the absence of extraneous solvent, and 20 gm. of silver oxide added over a period of 6 hours. As before, the methylated product was quantitatively removed from the silver salts with boiling methanol. After the third methylation the entire reaction mass crystallized when the solvents were completely removed.

The substance was recrystallized from ether by dissolving it in 20 cc. or more and evaporating the solvent on the water bath until less than 10 cc. of solvent remained, and cooling in the refrigerator. The average yield is about 8.0 gm., or 85 per cent of the theoretical, but by a remethylation of the mother liquors the yield becomes practically quantitative. The crystals form in solid, massive clumps and melt at 69–70° after one crystallization from ether. They are soluble in water, chloroform, benzene, the lower alcohols, and ether and sparingly soluble in petroleum ether. At the end of the third recrystallization from ether both the melting point and the rotation became constant. Melting point, 70.2–70.3°. Specific rotation

$$[\alpha]_{\mathrm{D}}^{28} = \frac{+4.95^\circ \times 100}{2 \times 1.742} = 142.1^\circ \text{ (in U.S.P. chloroform)}$$

The composition agreed with that of a pentamethylhexuronic acid.

5.193 mg. substance:	9.510 mg.	CO_2 and 3.520 mg. H_2O	
4.115 " "	: 18.505 "	AgI	
$C_{11}H_{20}O_7$.	Calculated.	C 49.97, H 7.6,	OCH_3 58.72
264.16	Found.	" 49.93, " 7.6,	" 59.36

Nitric Acid Hydrolysis and Oxidation of α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—α-Methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester (3.00 gm.) was simultaneously hydrolyzed and oxidized with 30 cc. of concentrated (*d* = 1.42) nitric acid. The reaction began at 65° and, as soon as the vigor of the reaction permitted, the temperature was raised to 95° and maintained there for 4 hours. The reaction mixture was then cooled and diluted with 50 cc. of water and transferred to a large Claisen distilling flask. Here the nitric acid was removed by evaporating the solution at 40° to a thick syrup under the vacuum of an efficient water pump, adding 5 to 10 cc. of water, and redissolving the syrup and concentrating again.

After repeating this evaporation cycle about twenty-five times the contents of the flask largely crystallized. The flask was evacuated an additional half hour at 10 mm. pressure and then for 3 more hours at less than 0.1 mm. The product from this treatment was free from nitric acid (nitron test). It was found that the viscous syrup surrounding the crystals was fairly soluble in dry ether, while the crystals did not dissolve appreciably on short contact with ether, so this method was used to effect their separation.

The crystals proved to be trimethoxymucic acid and the syrup was a mixture of oxidation products from which trimethoxy-*l*-araboglutaric acid and trimethoxymucic acid were isolated as their respective crystalline methylamides.

2,3,4-Trimethoxymucic Acid—The crystals obtained from the nitric acid hydrolysis and oxidation of α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester were quantitatively removed from the Claisen flask, by dissolving in acetone, transferred to a small Erlenmeyer flask, and the solution evaporated to about 5 cc. To this solution were added 100 cc. of ether and the solution placed in the refrigerator for 4 hours. At the end of this time well formed crystal rosettes had appeared. These were removed from the mother liquor, washed with ether, and when dry weighed 1.26 gm. and melted at 100–101°.

An additional 0.54 gm. was obtained from the mother liquor. After two more recrystallizations from acetone-ether, the melting point remained unchanged at 100–101° and the rotation was

$$[\alpha]_{\mathrm{D}}^{23} = \frac{+2.28^\circ \times 100}{2 \times 2.712} = +42.0^\circ \text{ (in acetone)}$$

Its composition corresponds to that of a trimethoxymucic acid.

4.002 mg. substance: 6.306 mg. CO_2 and 2.280 mg. H_2O
3.420 " " : 24.43 cc. 0.01 N $Na_2S_2O_3$

$C_9H_{16}O_8$.	Calculated.	C 42.84, H 6.4, OCH_3 36.91
252.12	Found.	" 42.96, " 6.4, " 36.92

Dimethyl Ester of 2,3,4-Trimethoxymucic Acid—2,3,4-Trimethoxymucic acid (1.2 gm.) was dissolved in 30 cc. of absolute methanol containing 3 per cent hydrogen chloride and gently refluxed overnight. The hydrogen chloride was removed with silver carbonate and the filtrate evaporated under reduced pressure to a solid crystalline mass. This was dissolved in an excess of ether, filtered, and concentrated to about 15 cc. Crystal plates formed and crystallization was completed in the refrigerator. Yield, 0.97 gm. A second crop of crystals weighing 0.30 gm. was obtained from the mother liquor.

The substance melts sharply at 100.5°. As this is practically the same melting point as that of the acid before esterification, a mixed melting point was made. That the two are entirely different was shown by the fact that the mixture melted at 79–82°. The non-identity of the two substances is substantiated by the elementary analysis and by the rotation. After two recrystallizations from ether the specific rotation was

$$[\alpha]_D^{24} = \frac{+1.28^\circ \times 100}{2 \times 2.217} = +28.9^\circ \text{ (in water)}$$

The composition corresponded to that of the dimethyl ester of a trimethoxymucic acid.

4.792 mg. substance:		8.290 mg.	CO_2 and 3.100 mg. H_2O
3.189 " "		: 13.305 "	AgI
	$C_{11}H_{20}O_8$.	Calculated.	C 47.12, H 7.2, OCH_3 55.37
	280.16	Found.	" 47.17, " 7.2, " 55.07

Dimethylamide of 2,3,4-Trimethoxymucic Acid—Dimethyl ester of 2,3,4-trimethoxymucic acid (0.25 gm.) was dissolved in 6 cc. of a concentrated solution of methylamine in dry methanol and kept at room temperature for 2 days. The solution was then concentrated to a solid crystalline mass under reduced pressure. It was recrystallized by dissolving it in 5 cc. of methanol and then adding 25 cc. of ethyl acetate. This mixture was evaporated to a volume of 15 to 18 cc. On cooling, clusters of long, stout needles formed. The product was filtered, washed with ethyl acetate, then ether, and dried. Yield, 0.24 gm. (practically quantitative).

The substance melted sharply at 207° and further recrystallization did not raise the melting point. The specific rotation was

$$[\alpha]_D^{22} = \frac{+0.41^\circ \times 100}{2 \times 1.630} = +12.6^\circ \text{ (in methanol)}$$

The analysis indicated that the compound is the dimethylamide of a trimethoxymucic acid.

3.809 mg. substance:	6.610 mg. CO_2 and 2.705 mg. H_2O		
4.198 " "	: 0.374 cc. N_2 (27° and 756 mm.)		
	$C_{11}H_{22}O_6N_2$.	Calculated.	C 47.45, H 8.0, N 10.07
	278.19	Found.	" 47.32, " 8.0, " 10.08

The diamide of 2,3,4-trimethoxymucic acid was also prepared, but it was found very unsuitable as a derivative for purposes of characterization.

Trimethoxy-l-Araboglutaric Acid from the Oxidation of α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—The non-crystalline fraction remaining after the removal of 2,3,4-trimethoxymucic acid from the mixture obtained by the nitric acid oxidation of α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester was evaporated under reduced pressure to free it from ether, and then dry methanol was added and subsequently removed under reduced pressure four times to eliminate traces of water.

The resulting syrup was dissolved in 20 cc. of dry methanol containing 3 per cent of dry hydrogen chloride and allowed to reflux overnight. The hydrogen chloride was then removed with silver carbonate and the methanol solution concentrated to a thin syrup. This was transferred to a microstill and distilled very slowly at 0.2 mm., the product being divided into four arbitrary fractions: A, 0.3 gm. distilling at a bath temperature of 100-120°; B, 0.4 gm., from 120-140°; C, 0.2 gm., from 140-150°; and a small still residue, D, remaining in the flask. All four fractions were undoubtedly mixtures, since there was no noticeable change in the rate of distillation as the temperature was gradually raised. The first three fractions were converted separately into the methylamides by the procedure recorded for the formation of 2,3,4-trimethoxymucic acid dimethylamide.

Fractions A and C yielded no pure crystalline chemical individual substances. Fraction A yielded a syrup and 0.13 gm. of a crystal mixture that contained both needles and more compact polyhedra and that melted at from 143-150°. After repeated recrystallizations from ethyl acetate and from methanol-ethyl acetate, two fractions were obtained, one melting at 151-161° and the other at 145-153°. Under the microscope both were still seen to be mixtures. Fraction C involved similar difficulties.

We were more successful with Fraction B in that we were able to isolate from it two pure substances: the dimethylamide of 2,3,4-trimethoxymucic acid and the dimethylamide of trimethoxy-*l*-araboglutaric acid. When a methanol-ethyl acetate solution of the methylamides of Fraction B was allowed to crystallize, 0.12 gm. of impure dimethylamide of 2,3,4-trimethoxymucic acid (m.p. 193–197°) separated. The mother liquor on further concentration (in effect, removing the methanol) and cooling yielded 0.13 gm. of impure dimethylamide of trimethoxyglutaric acid, m.p. 162–168°.

The dimethylamide of 2,3,4-trimethoxymucic acid was pure after two recrystallizations from methanol-ethyl acetate and melted at 206–207°. The mixed melting point with a pure authentic sample prepared directly from the crystalline methyl ester showed no depression in the melting point. The specific rotation was

$$[\alpha]_D^{25} = \frac{+0.21° \times 100}{1 \times 1.668} = +12.6° \text{ (in methanol)}$$

and the analysis substantiated the identity.

3.318 mg. substance: 0.296 cc. N_2 (28° and 759 mm.)
$C_{11}H_{22}O_6N_2$ (278.19). Calculated, N 10.07; found, N 10.09

The dimethylamide of trimethoxy-*l*-araboglutaric acid was pure after three recrystallizations from ethyl acetate and melted at 171–172°. A mixed melting point made with an authentic specimen (prepared by Dr. J. Compton in this laboratory) showed no depression. The specific rotation was

$$[\alpha]_D^{28} = \frac{+0.70° \times 100}{1 \times 1.163} = +60.2° \text{ (in water)}$$

which compares favorably with $[\alpha]_D^{18} = +59.9°$ and m.p. 172° reported by Haworth, Hirst, and Jones.[9]

The elementary analysis corresponds to that of the dimethylamide of trimethoxyglutaric acid.

5.313 mg. substance: 9.400 mg. CO_2 and 3.800 mg. H_2O
3.990 " " : 0.397 cc. N_2 (26° at 760 mm.)
$C_{10}H_{20}O_5N_2$. Calculated. C 48.35, H 8.1, N 11.29
248.18 Found. " 48.24, " 8.0, " 11.36

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[9] Haworth, W. N., Hirst, E. L., and Jones, D. I., *J. Chem. Soc.*, 2428 (1927).

α-Methyl-d-Galacturonamide—Hydrated α-methyl-*d*-galacturonide methyl ester (14.0 gm.) was dissolved in 20 cc. of dry methanol at 50° and the solution cooled to 20°. To this were added 100 cc. of a saturated solution of ammonia in dry methanol at 0° and the solution kept in the refrigerator overnight. A considerable portion of the product crystallized and was collected by filtering. A second crop of crystals was obtained by evaporating the filtrate to a volume of 50 cc. under reduced pressure, leaving in the refrigerator overnight, and then filtering the crystals that had formed. Total yield, 11.8 gm. (97 per cent of the theoretical). This was recrystallized by dissolving in 20 cc. of warm water, slowly adding 150 cc. of warm absolute ethanol with stirring, and then cooling in the refrigerator. M.p. 221–223°. After two more recrystallizations from water-ethanol, as above, the melting point and rotation were constant. M.p. 225–226°. The specific rotation was

$$[\alpha]_{\mathrm{D}}^{23} = \frac{+3.69^\circ \times 100}{2 \times 1.451} = +127.2^\circ \text{ (in water)}$$

It had the composition of α-methylgalacturonamide.

6 402 mg. substance: 0.378 cc. N_2 (25.5° at 762 mm.)
3.301 " " : 9.38 " 0.01 N $Na_2S_2O_3$
$C_7H_{13}O_6N$. Calculated. N 6.76, OCH_3 14.98
207.11 Found. " 6.77, " 14.69

Attempted "Weerman Degradation" of α-Methyl-d-Galacturonamide—The essential points in the method of Weerman[7] were followed. The reaction was very slow both in the cold and at room temperatures, but as the reactants were slowly heated to 60–65° the sodium hypochlorite was rapidly consumed, and after 30 minutes at that temperature it had disappeared entirely (as was shown by a negative test with starch-iodide paper). We were not able to isolate a degradation product, but we did succeed in recovering considerable quantities of a crystalline product which proved to be unchanged starting material.

Attempted "Hofmann Bromamide Degradation"[10] *of α-Methyl-d-Galacturonamide*—α-Methyl-*d*-galacturonamide (2.0 gm.) was dissolved in 5 cc. of water and 0.53 cc. (1.1 molecules) of bromine added and slowly warmed on the water bath. By the time the temperature had reached 60° the bromine had entirely dissolved.

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[10] Hofmann, A. W., *Ber. chem. Ges.*, **15**, 762 (1882).

A warm solution of 8.0 gm. of hydrated barium hydroxide in 40 cc. of water was then added dropwise and the following changes noted: the deep brown solution became yellow and a white precipitate of barium carbonate appeared. The reaction mixture was maintained at 60–70° for 2 hours, cooled to 15°, and then sulfuric acid added until the solution was just neutral to Congo red. Carbon dioxide was evolved and barium sulfate precipitated.

The precipitate was removed by centrifuging, and the resulting clear solution was freed in turn from bromine, silver, and barium by treating respectively with silver carbonate, hydrogen sulfide, and sulfuric acid, yielding a solution that was practically free from all inorganic matter. Upon evaporation of this solution under reduced pressure a considerable amount of crystalline material was obtained. This, on recrystallization from water-ethanol, yielded 0.9 gm. of crystal plates melting at 108–110° and after three more recrystallizations from the same solvents melting at 110–112°. It had the following composition.

5 514 mg. substance: 6 985 mg. CO_2 and 3.215 mg. H_2O

Halogen and nitrogen were both absent.

$C_7H_{16}O_9$ (244.12). Calculated, C 34.41, H 6.6; found, C 34.54, H 6.5

The specific rotation was

$$[\alpha]_D^{27} = \frac{+2.18^\circ \times 100}{1 \times 1.669} = +130.6^\circ \text{ (in water)}$$

The melting point, analysis, and specific rotation prove the product to be α-methyl-*d*-galacturonide dihydrate, reported by Niemann and Link[2] as having a melting point of 111.0–111.5° and $[\alpha]_D^{29} = +129.0^\circ \pm 1.0^\circ$ ($c = 1.3$ in water).

We wish to thank the Research Department of the California Fruit Growers Exchange, Ontario, California, for their generous gift of citrus pectin which has served as our source of galacturonic acid.

THE STRUCTURE OF MONOACETONE *d*-XYLULOSE

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The two ketopentoses (xylulose and ribulose) share with the two aldotetroses (erythrose and threose) the distinction of being the only unsubstituted true sugars in which the maximum size of the oxygen ring system (if any) is limited to 5 atoms (furanose).

In a former communication[1] we described the preparation of monoacetone *d*-xylulose and pointed out that, since it is nonreducing to boiling Fehling's solution and displays no mutarotation in water, the ketonic group must be linked with the acetone residue, necessitating the formulation of the sugar residue as a ring structure. The furanose ring was suggested, but there was no proof that a smaller ring system might not be present.

Prince and Reichstein[2] have recently oxidized monoacetone *d*-xylulose, obtaining the monoacetone derivative of a monobasic acid which they have named monoacetone *d*-xylosonic acid.

They assume, without evidence, that it has the structure (II) (and therefore assign to monoacetone *d*-xylulose the structure (I)). Actually, the possibility of the substance having either structure (III) or (IV) cannot reasonably be excluded without definite chemical proof.

From the practical standpoint, xylulose is of interest for at least three reasons. In the first place, *l*-xylulose is the pentose isolated from urine, in cases of pentosuria, by Levene and La Forge.[3] Secondly, *l*-xylulose is intimately related to *l*-xylosone, from which vitamin C (the lactone of 3-keto-*l*-gulonic (or *l*-talonic) acid) has been synthesized.[4] Thirdly, it has been shown that, unlike *d*-xylose, *d*-xylulose is readily converted to glycogen in the animal body.[5]

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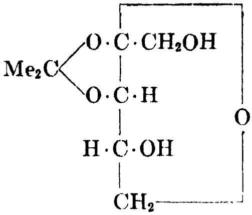
[1] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 603 (1934).

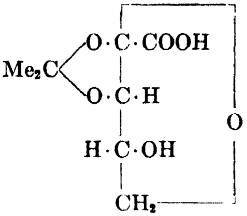
[2] Prince, R., and Reichstein, T., *Helv. chim. acta*, **20**, 101 (1937).

[3] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **18**, 319 (1914).

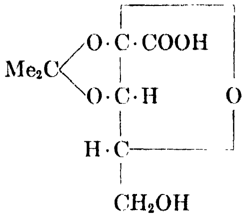
We are also interested in monoacetone *d*-xylulose as a possible starting material for the synthesis of thiomethyl *d*-xylulose, for comparison with the thiomethyl ketopentose obtainable from the adenine thiomethylpentoside present in yeast.



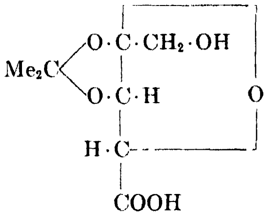
I



II



III



IV

In order to gain some insight into the structure of monoacetone *d*-xylulose it was decided to examine the behavior of its ditosyl ester towards sodium iodide dissolved in acetone.

It has been shown[6] that a tosyl group attached to the primary alcoholic group in glucose derivatives is readily substituted by iodine on treatment with this reagent at 100° during 2 hours. The applicability of the reaction has since been extended to the aldopentofuranoses by Levene and Raymond,[7] but they found that more prolonged treatment (during 6 hours or more) is necessary for such substances and that the rate of reaction is influenced by the presence of other substituents.

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[4] Reichstein, T., *et al.*, *Nature*, **131**, 280 (1933); *Helv. chim. acta*, **16**, 1019 (1933). Haworth, W. N., *et al.*, *J. Chem. Soc.*, 1419 (1933).

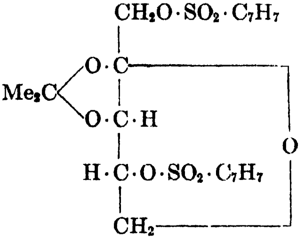
[5] Larson, H. W., Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., and Sawyer, S. D., *J. Biol. Chem.*, **117**, 719 (1937).

[6] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

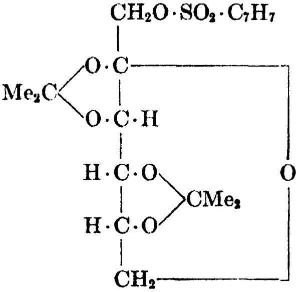
[7] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

In the same way it might be expected that a *6-tosyl* ketohexose derivative would react with sodium iodide but, so far as we are aware, no study of the behavior of *1-tosyl* ketohexose derivatives towards this reagent has been made. It is well known that the reactivity of the primary hydroxyl group *adjacent* to the keto group is profoundly influenced by that group and is consequently quite different from that of the primary hydroxyl *remote* from the reducing group.

We have now found that 1-tosyl 2,3,4,5-diacetone fructose is unaffected by sodium iodide in acetone during 2 hours at 100°. *Ditosyl monoacetone d-xylulose* is also unaffected by the same treatment. This evidence indicates that there is no tosyl group attached at carbon atom (5) of the chain, but cannot be regarded as a rigorous proof of the position of the tosyl groups and thence of the structure of the substance. Recourse was therefore had to a study of the methylated derivatives of monoacetone *d*-xylulose.



1,4-Ditosyl monoacetone
d-xylulose



1-Tosyl β-diacetone
d-fructose

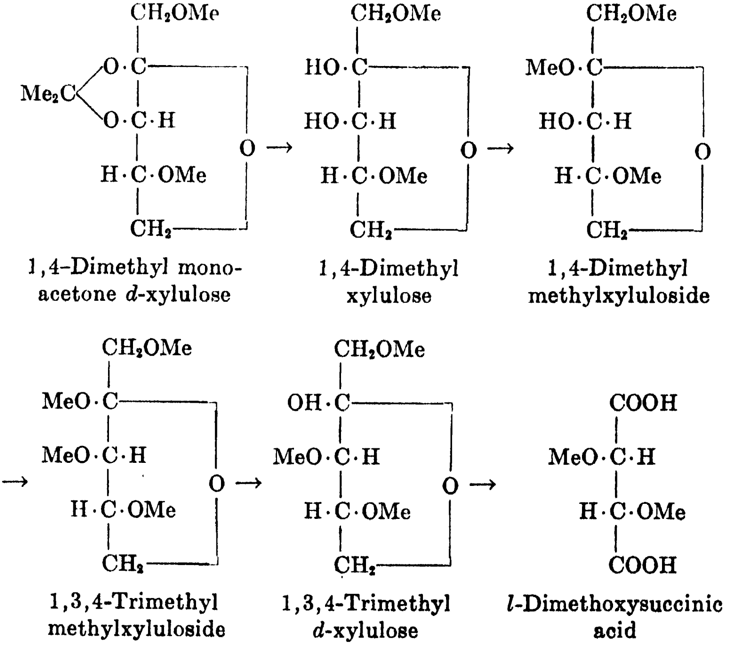
Crystalline monoacetone *d*-xylulose was completely methylated by means of Purdie's reagents to give *dimethyl monoacetone d-xylulose.* On hydrolysis of the acetone residue by means of aqueous oxalic acid solution (0.2 N) a crystalline *dimethyl xylulose,* which was strongly reducing to boiling Fehling's solution, was isolated in practically quantitative yield. The reducing group at position (2) is therefore protected by the acetone residue in the monoacetone derivative, but free in the dimethyl sugar.

The new dimethyl xylulose condensed at room temperature with methyl alcohol (containing hydrogen chloride) with the extreme ease characteristic of furanose sugars. The resulting *dimethyl methylxyluloside* was now methylated by means of Purdie's reagents, forming *trimethyl methylxyluloside.*

The structure of this as *1,3,4-trimethyl methylxylulofuranoside* was established in the following manner. On oxidation with concentrated nitric acid an acid was formed which, on further oxidation by means of acid permanganate, gave crystalline *l*-dimethoxysuccinic acid directly (without intermediate isolation of a dimethyl tetronolactone).

From these results it follows that monoacetone *d*-xylulose is to be designated 2,3-isopropylidene *d*-xylulofuranose.

It is of interest that the isopropylidene group in derivatives of monoacetone xylulose is more resistant towards acid hydrolysis than is the glycosidic methoxyl group in derivatives of methylxyluloside.



1,4-Dimethyl monoacetone *d*-xylulose

1,4-Dimethyl xylulose

1,4-Dimethyl methylxyluloside

1,3,4-Trimethyl methylxyluloside

1,3,4-Trimethyl *d*-xylulose

l-Dimethoxysuccinic acid

EXPERIMENTAL

Preparation of 1,4-Ditosyl Monoacetone d-Xylulose—2.5 gm. of dry, recrystallized monoacetone *d*-xylulose were dissolved in 15 cc. of dry pyridine, 5.5 gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved.

After standing overnight at room temperature, with the exclusion of atmospheric moisture, 1 cc. of water was added to the pale brown solution. The resulting solution was kept at room temperature during 30 minutes and the product isolated in the usual manner,[8] giving 6.5 gm. of a colorless gum. The substance was dissolved in 25 cc. of absolute ethanol or methanol (at 40°) and, on cooling to room temperature, it rapidly crystallized.

It had a melting point of 71–73° and the following composition.

4.022 mg. substance: 7.810 mg. CO_2 and 1.895 mg. H_2O
7.122 " " : 6.624 " $BaSO_4$

$C_{22}H_{26}O_9S_2$.	Calculated.	C 52.98, H 5.3, S 12.87
	Found.	" 52.95, " 5.3, " 12.78

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.15° \times 100}{2 \times 1.202} = +6.3° \text{ (in acetone)}$$

Action of Sodium Iodide on Ditosyl Monoacetone d-Xylulose—A mixture of 0.5 gm. of dry, recrystallized ditosyl monoacetone *d*-xylulose with 0.6 gm. of sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution remained colorless and no crystalline material separated out during this treatment.

However, after heating at 100° during 8 hours some crystalline sodium *p*-toluene sulfonate settled out on cooling. The product was isolated in the usual manner, giving a colorless syrup which was dissolved in absolute methanol. On cooling, unchanged starting material (amounting to over 50 per cent of the original weight taken) crystallized out. After filtration, the mother liquor yielded a colorless syrup which contained iodine but which was so contaminated by unchanged starting material that the iodo derivative could not be obtained in crystalline form.

[8] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **105**, 419 (1934).

Preparation of 1-Tosyl 2,3,4,5-Diacetone d-Fructose[9]—10 gm. of dry, recrystallized β-diacetone fructose were dissolved in 60 cc. of dry pyridine, 8.1 gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved.

After standing overnight at room temperature, with the exclusion of atmospheric moisture, 1 cc. of water was added to the pale pinkish yellow solution. The resulting solution was kept at room temperature during 30 minutes and the product then isolated in the usual manner, giving 15.9 gm. of a colorless gum. The substance was dissolved in 25 cc. of absolute ethanol (at 40°) and, on cooling to room temperature, it rapidly crystallized.

It had a melting point of 83° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.76° \times 100}{2 \times 1.470} = -25.9° \text{ (in absolute ethanol)}$$

Its composition was as follows:

4.219 mg. substance: 8.510 mg. CO_2 and 2.395 mg. H_2O
6.235 " " : 3.582 " $BaSO_4$
$C_{19}H_{26}O_8S$. Calculated. C 55.04, H 6.3, S 7.74
Found. " 55.05, " 6.4, " 7.81

Action of Sodium Iodide on 1-Tosyl 2,3,4,5-Diacetone d-Fructose—A mixture of 0.5 gm. of 1-tosyl β-diacetone *d*-fructose with 0.4 gm. of sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution remained colorless and no crystalline material separated out during this treatment.

Even after heating at 100° during 8 hours the solution remained colorless and no crystalline material separated out on cooling.

Preparation of Dimethyl Monoacetone d-Xylulose—5 gm. of dry, recrystallized monoacetone *d*-xylulose were dissolved in 5 cc. of cold acetone and 50 cc. of methyl iodide were added, the solution remaining clear and colorless. 5 gm. of silver oxide were added and the solution was boiled gently under a reflux, with mechanical stirring. Four further portions (5 gm. each) of silver oxide were added at intervals of 30 minutes and the reaction was allowed to proceed for a total of 5 hours.

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[9] Ohle, H., and Koller, I., *Ber. chem. Ges.*, **57**, 1566 (1924).

The product was isolated in the usual manner[8] (dry ether being used as solvent) and remethylated with the same amounts of Purdie's reagents (*without* the addition of acetone).

The resulting syrup (5.6 gm.) was distilled, giving a main fraction (5 gm.) boiling at 47° at 0.1 mm. (bath temperature, 51°) which had $n_D^{25} = 1.4371$. A second fraction (0.3 gm.), which was not quite fully methylated, had $n_D^{25} = 1.4390$. The still residue was of negligible weight.

The main fraction was a colorless, extremely mobile liquid which had the following composition.

5.408 mg. substance: 10.890 mg. CO_2 and 4.090 mg. H_2O
5.952 " " : 12.860 " AgI
$C_{10}H_{18}O_5$. Calculated. C 55.01, H 8.3, OCH_3 28.44
Found. " 54.91, " 8.4, " 28.52

Its specific rotation was as follows:

$$[\alpha]_D^{24} = \frac{-0.33° \times 100}{2 \times 1.308} = -12.6° \text{ (in acetone)}$$

Hydrolysis of Dimethyl Monoacetone d-Xylulose—The course of hydrolysis at 65° of a 2.294 per cent solution of dimethyl monoacetone *d*-xylulose in 0.2 N aqueous oxalic acid (having initial $[\alpha]_D^{25} = +3.2°$) was studied polarimetrically as previously described for monoacetone *d*-xylulose.[10] After 2 hours the substance was only hydrolyzed to the extent of 48 per cent and 10 hours were required for complete hydrolysis, the solution then having $[\alpha]_D^{25} = -17.3°$ (calculated as dimethyl pentose). The intermediate values observed were 71.4 (4 hours), 85.7 (6 hours), and 92.9 per cent (8 hours). No decomposition occurred, since the final rotation remained unchanged after treatment during 24 hours at 65°.

Accordingly, a solution of 5.0 gm. of dimethyl monoacetone *d*-xylulose in 218 cc. of 0.2 N oxalic acid solution was kept in a bath at 65° for at least 10 hours. It was then cooled in ice to room temperature and the product isolated as described for xylulose from monoacetone xylulose,[10] giving 4.0 gm. of a perfectly colorless, thick syrup. The product was dissolved in about 10 cc. of dry ether and pentane was added to faint opalescence, whereupon dimethyl xylulose crystallized in clusters of long, fine needles. In a few minutes it had set to an almost solid mass.

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[10] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **115**, 731 (1936).

The substance was readily distilled (without decomposition) under a high vacuum. It had a boiling point of 88° at 0.2 mm. (bath temperature, 105°) and was collected as a colorless, fairly mobile syrup (having $n_D^{25} = 1.4630$) which crystallized to a solid mass again on nucleation and then had a melting point of 48–49°. It was strongly reducing to boiling Fehling's solution and had a transitory bitter-sweet taste.

Its composition was as follows:

5.102 mg. substance: 8.811 mg. CO_2 and 3.646 mg. H_2O
4.184 " " : 10 950 " AgI
$C_7H_{14}O_5$. Calculated. C 47.16, H 7.9, OCH_3 34.84
Found. " 47.09, " 8.0, " 34.57

It had the following specific rotation.

$$[\alpha]_D^{26} = \frac{-0.54^\circ \times 100}{2 \times 1.010} = -26.7^\circ$$ (in absolute methanol, 2 min. after admixture)

changing to the constant value $[\alpha]_D^{26} = -16.3^\circ$ (30 minutes).

Preparation of Dimethyl Methylxyluloside—Crystalline dimethyl xylulose condensed rapidly at 26° with methanol containing 1 per cent of hydrogen chloride. The specific rotation at various time intervals was as follows: −11.2° (4 minutes), −9.8° (5 minutes), −14.1° (10 minutes), −15.6° (20 minutes), and −16.6° (30 minutes), constant thereafter.

Accordingly, 2.5 gm. of crystalline dimethyl xylulose were dissolved in 250 cc. of cold methyl alcohol containing 2.5 gm. of hydrogen chloride. The solution was kept at room temperature (26°) during 60 minutes and then dry silver carbonate was added until the solution was neutral to Congo red.

The mixture was filtered and the silver salts extracted three times with boiling dry ether. The filtrate and extracts were combined and evaporated to dryness under diminished pressure. The syrupy product was dissolved in dry ether, a trace of insoluble silver salt filtered off, and the filtrate reevaporated to dryness, giving a perfectly colorless, mobile syrup (weight, 2.7 gm.) which was distilled under a high vacuum.

Three fractions were collected (b.p., 61–64° at 0.25 mm., with bath temperature 70–72°) as colorless, quite mobile liquids with the following properties.

Fraction 1. (0.94 gm.) n_D^{25} = 1.4479; $[\alpha]_D^{20}$ = −18.6° (in acetone, c = 1.24)
" 2. (1.44 ") " = 1.4482; " = −7.0° (" " " = 1.32)
" 3. (0.32 ") " = 1.4485; " = +4.3° (" " " = 1.29)

Though there was no significant difference in boiling point (or refractive index) for each fraction, observation of the specific rotation revealed the partial separation of the α from the β isomer of the glycoside and there is little doubt but that, by repeated fractionation, a pure specimen of each isomer could be obtained.

The composition of each fraction corresponded with that of a dimethyl methylpentoside, *e.g.* the first fraction had the following composition.

5.697 mg. substance: 10.404 mg. CO_2 and 4.290 mg. H_2O
5.511 " " : 20.104 " AgI
$C_8H_{16}O_5$. Calculated. C 49.97, H 8.4, OCH_3 48.45
Found. " 49.80, " 8.4, " 48.15

Methylation of Dimethyl Methylxyluloside—4.0 gm. of dimethyl methylxyluloside were dissolved in 50 cc. of methyl iodide and treated with 25 gm. of silver oxide (added in portions of 5 gm., every 30 minutes), the mixture being boiled gently under a reflux, with mechanical stirring. The reaction was allowed to proceed for a total of 5 hours.

The product was isolated in the usual manner, giving a colorless, very mobile syrup (4.4 gm.) which was distilled under a high vacuum. No attempt was made to separate the α from the β form of the glycoside. It boiled at 52° at 0.25 mm. (bath temperature, 55–56°) and was collected as a colorless, very mobile liquid having n_D^{25} = 1.4368 and the following composition.

3.281 mg. substance: 6.300 mg. CO_2 and 2.600 mg. H_2O
3.600 " " : 16.390 " AgI
$C_9H_{18}O_5$. Calculated. C 52.40, H 8.8, OCH_3 60.21
Found. " 52.36, " 8.9, " 60.09

Other workers[11] state that "in view of the highly volatile nature of the trimethyl methylpentosides, the distillations were carried out at a pressure not lower than about 14 mm." In the present case we found that there was no loss of material when the distillation was performed at 0.25 mm., provided that the distillation was slow and the receiver was cooled in ice. A trap, cooled in solid carbon dioxide-chloroform, was connected between the receiver and the high vacuum pump, but no material collected there.

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[11] Haworth, W. N., Hirst, E. L., and Oliver, E., *J. Chem. Soc.*, 1917 (1934).

Hydrolysis of Trimethyl Methylxyluloside—The course of hydrolysis at 65° of a 2.344 per cent solution of trimethyl methylxyluloside in 0.2 N aqueous oxalic acid (having initial $[\alpha]_D^{25} = +1.3°$) was studied polarimetrically, as previously described for the hydrolysis of monoacetone *d*-xylulose.[10] After 30 minutes, hydrolysis was practically complete. The course of the hydrolysis was as follows: 37.8 (5 minutes), 70.8 (15 minutes), 84.1 (20 minutes), 93.0 (25 minutes), 98.7 (30 minutes), and 100 per cent (60 minutes). No decomposition occurred, since the final rotation ($[\alpha]_D^{25} = -14.4°$, calculated as trimethyl pentose) remained unchanged after treatment during 18 hours at 65°.

Accordingly, a solution of 1.17 gm. of trimethyl methylxyluloside in 50 cc. of 0.2 N oxalic acid solution was kept in a bath at 65° for 60 minutes. It was then cooled in ice to room temperature, and the product isolated in the usual manner and distilled under a high vacuum. It had a boiling point of 64° at 0.25 mm. (bath temperature, 72°) and was collected as a colorless, quite mobile liquid having $n_D^{25} = 1.4458$, which was strongly reducing to boiling Fehling's solution.

Its composition was as follows:

3.623 mg. substance: 6.667 mg. CO_2 and 2.695 mg. H_2O
4.302 " " : 40.447 cc. 0.01 N thiosulfate

$C_8H_{16}O_5$. Calculated.	C 49.97,	H 8.4,	OCH_3 48.45
Found.	" 50.18,	" 8.3,	" 48.38

The distilled, syrupy substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.34° \times 100}{2 \times 1.216} = -14.0° \text{ (in absolute methanol)}$$

Oxidation of Trimethyl Xylulose with Nitric Acid—1 gm. of trimethyl xylulose (or 1.07 gm. of trimethyl methylxyluloside) was dissolved in 10 cc. of concentrated nitric acid ($d = 1.42$) at 25°

and the temperature raised until, at 59°, a vigorous action ensued. The temperature was maintained at 59–60° during 15 minutes and then gradually raised until, after a further 75 minutes, it had reached 95°.

The solution was now cooled and evaporated to dryness under diminished pressure at 50° with a distilling flask provided with a trap (to prevent loss by splashing) at the point of attachment of the side arm. A portion (10 cc.) of water was added and the solution evaporated to dryness. The process of adding water in portions of 10 cc. and evaporating to dryness after each addition was repeated until a sample of the distillate gave no precipitate on addition of a few drops of a 10 per cent solution of nitron in 5 per cent aqueous acetic acid, together with a few drops of dilute sulfuric acid, and allowing to stand. In this manner, the whole of the nitric acid was removed in some 4 hours (as compared with the customary period, reported in the literature, of several days).

The product, consisting of a colorless syrup containing a small amount of crystalline material, was dried by adding benzene and evaporating to dryness, the procedure being repeated several times. It was now dissolved in dry ether, a trace of insoluble material filtered off, and the filtrate evaporated to dryness in a tared dish. It was dried overnight, over phosphorus pentoxide and potassium hydroxide, in a vacuum desiccator at room temperature (27°) and 0.05 mm. The colorless syrup, containing a few colorless, rectangularly shaped crystals, weighed 0.89 gm. (theoretical yield, 0.93 gm.).

Esterification and Methylation of Dimethyl Xylosonic Acid—The crude dimethyl xylosonic acid (0.89 gm.) was esterified in the following manner. It was first freed from traces of water by dissolving in absolute methanol and evaporating the solution to dryness, this procedure being repeated three times.

The syrup was then dissolved in 25 cc. of absolute methanol containing 3 per cent of dry hydrogen chloride and the solution boiled, under a reflux condenser closed by a calcium chloride tube, during 6.5 hours.

The solution was now cooled in ice and rendered neutral to Congo red by the addition of finely powdered silver carbonate. The mixture was filtered and the silver salts well washed with dry ether. The filtrate and washings were combined and evaporated to dryness under diminished pressure. The product was dissolved in dry ether, filtered from a trace of insoluble impurity, and the filtrate evaporated to dryness, giving a pale yellow syrup (weight, 0.8 gm.) which was distilled at high vacuum.

Only one fraction was collected. This boiled at 89–90° at 0.20 mm. (bath temperature, 99–100°) and weighed 0.73 gm. It had $n_D^{25} = 1.4424$ and was reducing to boiling Fehling's solution.

It had the following composition.

4.422 mg. substance: 7.694 mg. CO_2 and 2.600 mg. H_2O
4.892 " " : 46 45 cc. 0.01 N thiosulfate

		C	H	OCH_3
(*a*) $C_8H_{14}O_6$.	Calculated.	46.58	6.9	45.16
(*b*) $C_9H_{16}O_6$.	"	49.06	7.3	56.37
(*c*) $C_8H_{14}O_6$.	"	46.58	6 9	60.21
	Found.	47.44	6.6	49.10

The product was apparently a mixture of the methyl ester of dimethyl xylosonic acid (*a*) with its "methylglycoside" (*b*), but, as shown later, it also contained a little dimethyl *l*-dimethoxysuccinate (*c*).

It was therefore subjected to complete methylation by means of Purdie's reagents. The product was isolated in the usual manner and distilled at high vacuum. The first fraction (0.2 gm.) boiling at 70–72° at 0.1 mm. (bath temperature, 84–85°) had $n_D^{25} = 1.4371$. It was dissolved in 5 cc. of absolute methanol saturated with dry ammonia. After standing in the refrigerator during 2 days, star-like clusters of long, colorless needles separated. The product was filtered off, washed with ethyl acetate, and dried. It had a melting point of 270° (with previous darkening[12] at 250°) and its composition corresponded with that of the diamide of a dimethoxysuccinic acid.

4.603 mg. substance: 6.907 mg. CO_2 and 2.910 mg. H_2O
5.389 " " : 0.745 cc. N_2 (755 mm. at 28°)

		C	H	N
$C_6H_{12}O_4N_2$.	Calculated.	40.88	6.9	15.91
	Found.	40.91	7.1	15.56

Oxidative Degradation of Dimethyl Xylosonic Acid by Means of Acid Permanganate—The method employed was essentially that of Haworth and Learner[13] except that, in order to avoid the presence of any manganese salt in the final product, the use of carbon dioxide as a neutralizing agent was eliminated.

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[12] Haworth, W. N., and Jones, D. I., *J. Chem. Soc.*, 2349 (1927).

Syrupy crude dimethyl xylosonic acid (0.8 gm.) was dissolved in 22 cc. of 0.5 N sulfuric acid and the solution diluted to 50 cc. with distilled water. An aqueous solution of barium permanganate (approximately 4 per cent) was now slowly added, with shaking, to the solution at room temperature until manganese oxides began to be precipitated.

A cold, saturated aqueous solution of barium hydroxide was then added until the solution was alkaline to thymolphthalein and further addition of barium hydroxide to a centrifuged test portion gave no brown precipitate.

The mixture was centrifuged and the precipitate washed by centrifuging with several changes of water. The solution and washings were united and N sulfuric acid was added dropwise until only a trace of barium remained in solution. The mixture was centrifuged and the precipitate washed with water.

The solution and washings were combined and evaporated to dryness under diminished pressure. The product was dissolved in dry ether, a trace of insoluble barium salt filtered off, and the filtrate evaporated to dryness, giving a solid mass of colorless crystals (weight, 0.5 gm.). It was recrystallized by dissolving in the minimum of boiling, dry ether, adding an equal volume of pentane, and cooling. On nucleating and stirring it separated in beautiful colorless crystals having a melting point of 151° and the the following specific rotation.

$$[\alpha]_{\mathrm{D}}^{26} = \frac{-1.55^\circ \times 100}{2 \times 1.126} = -68.8^\circ \text{ (in water)}$$

It had the following composition.

4.912 mg. substance: 7.305 mg. CO_2 and 2.688 mg. H_2O
4.200 " " : 27.98 cc. 0.01 N thiosulfate
$C_6H_{10}O_6$. Calculated. C 40.43, H 5.7, OCH_3 34.84
Found. " 40.55, " 6.1, " 34.44

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[13] Haworth, W. N., and Learner, A., *J. Chem. Soc.*, 619 (1928). Avery, J., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 2308 (1927)

A NOTE ON THE ACRIDINE SALTS OF "YEAST" AND "MUSCLE" ADENYLIC ACIDS

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(Received for publication, July 2, 1937)

In the course of an investigation dealing with the preparation of 5-phosphoadenosine from adenosine, occasion was taken to prepare the acridine salt of natural "muscle" adenylic acid, with a view to utilizing the same method later for the precipitation of the synthetic substance.

The product obtained from the natural acid had a composition quite different from that recorded by Wagner-Juaregg.[1] Whereas he gives the formula $C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P \cdot H_2O$, the acridine salt now described had a composition agreeing with the formula $C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$.

A similar product, prepared by combining "yeast" adenylic acid with acridine, also had a composition indicating the formula $C_{18}H_9N \cdot 2C_{10}H_{14}O_7N_5P$.

EXPERIMENTAL

Acridine Salt of Authentic "Muscle" Adenylic Acid—The acridine salt was prepared as described by Wagner-Juaregg.[1] After being recrystallized twice from water, it was dried in a vacuum desiccator over phosphorus pentoxide, at 0.1 mm. and room temperature, during 2 days.

The lemon-yellow product had a melting point of 217–218°, with previous slight darkening in color. Its composition was as follows:

4.626 mg. substance: 7.785 mg. CO_2 and 1.805 mg. H_2O
4.310 " " : 0.652 cc. N_2 (762 mm. at 25°)
4.510 " " : 21.895 mg. ammonium phosphomolybdate
$C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$. Calculated. C 45.34, H 4.3, N 17 64, P 7.10
Found. " 45.89, " 4.4, " 17.38, " 7.05

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[1] Wagner-Juaregg, T., *Z. physiol. Chem.*, **239**, 188 (1936).

Wagner-Juaregg contended that the substance had the formula

$C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P \cdot H_2O$. Calculated. C 50.71, H 4.63, N 15.44, P 5.70
$C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P$. " " 52.45, " 4.41, " 15.97, " 5.90

The dried acridine salt had the following specific rotation

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-0.25° \times 100}{2 \times 0.538} = -23.2° \text{ (in 10\% HCl)}$$

5 minutes after admixture. Recalculated as free adenylic acid, this gives a value of $[\alpha]_{\mathrm{D}}^{25} = -29.2°$, which supports our formulation of the substance. Embden and Schmidt[2] state that "muscle" adenylic acid has $[\alpha]_{\mathrm{D}}^{20} = -26.0°$ (in 10 per cent hydrochloric acid), but the observed data which they record give the value $[\alpha]_{\mathrm{D}}^{20} = -31.0° (\pm 1.0°)$.

After 14 days at room temperature, this solution had $[\alpha]_{\mathrm{D}}^{25} = +13.9°$, which, recalculated as 5-phosphoribose, gives a value of $[\alpha]_{\mathrm{D}}^{25} = +26.5°$, affording further verification of the above formulation. From the data recorded by Levene and Stiller,[3] the specific rotation of 5-phosphoribose is calculated to be $[\alpha]_{\mathrm{D}}^{26} = +25.6°$ to $+26.0°$.

Acridine Salt of Authentic "Yeast" Adenylic Acid.—To a solution of 300 mg. of yeast adenylic acid in 17 cc. of boiling distilled water was added a solution of 154.8 mg. of twice recrystallized acridine in 2 cc. of absolute ethanol, giving a deep yellow solution but no crystals. The solution was now evaporated to dryness in a vacuum desiccator, giving a mass of yellow crystals. The product was twice recrystallized, first from 5 cc. of boiling distilled water and then from 6 cc. of the same solvent. The lemon-yellow crystalline product was dried in a vacuum desiccator over phosphorus pentoxide, at 0.1 mm. and room temperature during 3 days.

The dried substance had a melting point of 183–184° (with no previous darkening) and the following composition.

4.511 mg. substance: 7.505 mg. CO_2 and 1.950 mg. H_2O
4.520 " " : 0.688 cc. N_2 (775 mm. at 26°)
3.895 " " : 18.915 mg. ammonium phosphomolybdate
$C_{33}H_{37}O_{14}N_{11}P_2$. Calculated. C 45.34, H 4.3, N 17.64, P 7.10
Found. " 45.37, " 4.8, " 17.71, " 7.05

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[2] Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929).

[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

Observation of the specific rotation of this preparation afforded confirmation of the above formulation. It had

$$[\alpha]_D^{25} = \frac{-0.30° \times 100}{2 \times 0.524} = -28.6° \text{ (in 10\% HCl)}$$

10 minutes after admixture. Recalculated as free adenylic acid, this gives a value of $[\alpha]_D^{25} = -35.9°$. Embden and Schmidt[2] record $[\alpha]_D^{20} = -36.5°$ for "yeast" adenylic acid in this solvent.

THE LIPID ANALYSIS OF HUMAN THORACIC DUCT LYMPH*

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(Received for publication, June 14, 1937)

As has been pointed out by Boyd (1), it is becoming increasingly evident that a satisfactory study of fat metabolism requires that all the fatty constituents be taken into consideration. There is lacking, however, a unified method of analysis whereby one can separate all the lipids in a single sample of tissue.

With the procedures now in use, if one wishes to make a complete differential analysis, it is necessary to take aliquots of a solution of the total lipids and determine one, or perhaps two, constituents on each aliquot. Also, to determine both the quantity and the nature of a single constituent, such as the amount and iodine number of neutral fat fatty acids, it is necessary to take two such aliquots. Such a procedure was adopted by Boyd (1).

This has several objectionable features. The first of these is the manipulative complications.

The second objection is the determination of one constituent in the presence of another. This is best exemplified by the determination of neutral fat fatty acid in the presence of cholesterol by the use of the oxidative procedure. When this is done, it is necessary to subtract the number of ml. of 0.1 N potassium dichromate required to oxidize the cholesterol from the number of ml. required to oxidize both the cholesterol and the fatty acids. Not only does this necessitate the additional calculation, but any error in the determination of cholesterol becomes an error in the determination of fatty acids. In the analysis of lymph, as given in this paper, neutral fat fatty acids were determined in the presence of cholesterol. Since this paper has been completed, however, methods have been developed for the analysis of nerve and blood lipids whereby free and bound cholesterol and neutral fat fatty acids are separated before their determination. The results of that work are in preparation for publication.

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* The data presented in this paper formed part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the Ohio State University, 1936.

A third objection is the waste of the constituent not determined. This may not be of serious concern in most cases, but there are instances in which only small amounts of material are available and all of each constituent is necessary for a determination.

A further deficiency in the present micromethods is the lack of any procedure for the determination of the mean molecular weight of the fatty acids.

EXPERIMENTAL

The human lymph used in this study was made available through the cooperation of Dr. A. Strauss of Mt. Sinai Hospital in Cleveland, who has published a complete account of the injury to the thoracic duct from which the lymph was obtained (2).

It should be noted that the analyses reported in the present paper were of lymph aspirated from 24 to 44 days after the lymph had begun to flow from the injured thoracic duct and after 20.8 to 46.7 liters had been withdrawn.

It must also be borne in mind that the patient was on a rigid low fat diet as soon as the nature of her injury was ascertained.

Method

Extraction—Since this method of partition depends upon the separation of the lipid constituents rather than upon their determination in an aliquot of a solution of the total lipid, it is necessary that the total lipid of the sample be isolated. The almost universal procedure of Bloor of pipetting a measured quantity of fluid tissue into a 3:1 mixture of alcohol and ether cannot be readily adapted to this end.

The method finally adopted was a modified Roese-Gottlieb procedure for the extraction of fat from milk products.

10 ml. of lymph were pipetted into a 250 ml. glass-stoppered, graduated cylinder containing 11 ml. of 95 per cent alcohol and 1 ml. of 1:3 sulfuric acid. 2.5 volumes of ethyl ether (24 ml.) and

2.5 volumes of petroleum ether, b.p. 30–60°, were added in that order, the cylinder being vigorously shaken after each addition. After settling, the ether layer was removed by means of a siphon arrangement similar to that of a wash bottle, the end of the tube in the ether being bent up. The ether solution was transferred to a 150 ml. Erlenmeyer flask and the ether evaporated to a small volume on the steam bath.

The extraction with the ethers was repeated twice more, a little alcohol being added each time to keep the water and alcohol layer at 22 ml. After each extraction the ether solution was added to the Erlenmeyer flask, and evaporated as before. After the final extraction it was carefully evaporated to dryness, and weighed.

The fat was removed from the flask with small amounts of ethyl and petroleum ethers, alternately, the extracts being poured into a 15 ml. conical bottom centrifuge tube and each extract evaporated in its turn to about 0.5 ml. by immersing the tube in hot water. A short piece of melting point tube fused to a thin glass rod was used as an antibumper. After the final extraction the Erlenmeyer flask was again dried in the desiccator and weighed and total fat thus determined by difference.

Separation of Phospholipid—After about five extractions the petroleum ether solution was boiled to about 0.5 ml., 3 drops of a saturated solution of strontium chloride in 95 per cent alcohol were added, and then 5 ml. of acetone. Strontium chloride was used instead of magnesium chloride, as it has been shown that this salt more completely precipitates phospholipid in acetone solution (3). The mixture was well stirred with the same glass rod used as antibumper, the melting point tube having been crushed. The tube was allowed to stand 1 hour and then centrifuged at 1500 R.P.M. for about 3 minutes.

The supernatant solution, which contained the free and bound cholesterol and neutral fat, was transferred to a 50 ml. volumetric flask and the residue washed twice with 2 ml. volumes of acetone. The washings, after centrifuging, were added to the volumetric flask which was then made up to volume.

Determination of Phospholipids—The phospholipid salt was dissolved in moist, peroxide-free ether and made up to volume in a 25 ml. volumetric flask. 5 ml. were transferred to an oxidation flask and a determination made according to Boyd (4). 3 ml. of 0.1 N $K_2Cr_2O_7$ were considered as equivalent to 1 mg. of phospholipid.

Determination of Phospholipid Fatty Acids—The remaining 40 ml. of the ether solution of the phospholipid were transferred to a 50 ml. Erlenmeyer flask, the ether evaporated, and the lipid dissolved in 5 ml. of 95 per cent alcohol. 0.1 ml. of a 50 per cent potassium hydroxide solution was added and the solution heated on the steam bath for about an hour. The alcohol was then evaporated until there was no longer any odor of the vapor. 1 ml. of 20 per cent phosphoric acid was added and heated for a few minutes on the steam bath. The fatty acids were then extracted five or six times with petroleum ether, b.p. 30–60°. The extracts were transferred to a 50 ml. volumetric flask which was then made up to volume. These extracts were not clear, but if the flasks were centrifuged for 5 to 10 minutes at 1500 R.P.M., the cloudy precipitate would settle and adhere to the bottom.

10 ml. of this solution were pipetted into a 125 ml. glass-stoppered Erlenmeyer flask and the acids oxidized according to Bloor (5). 20 ml. of this solution were slowly pipetted into a 75 × 22 mm. weighing bottle immersed in hot water. The bottle contained a few grains of silica sand as antibumper. These acids were titrated with 0.01 N sodium hydroxide by a method described below. The remaining 20 ml. were transferred to another 125 ml. glass-stoppered Erlenmeyer flask for the determination of iodine number.

The factor 1.4 was used to calculate phospholipid from phospholipid fatty acids.

Determinations of Neutral Fat Fatty Acids—40 ml. of the acetone solution containing the cholesterol and neutral fat were evaporated to dryness in a 50 ml. Erlenmeyer flask, saponified, acidified, extracted, and divided in the same manner as the phospholipid fatty acids. It was necessary to correct for cholesterol in calculating quantity and iodine number. Triglycerides were calculated by multiplying by the factor 1.04.

Determination of Total Cholesterol—A 5 ml. aliquot of the acetone solution was saponified and extracted similarly. The extract, however, was transferred to a 50 ml. Erlenmeyer flask and evaporated to dryness. It was then redissolved in 5 ml. of petroleum ether and 5 ml. of a 0.2 per cent solution of digitonin in 50 per cent alcohol were added. The mixture was evaporated almost to dryness on the steam bath.

From this point the determination was precisely according to Boyd (4).

Determination of Free Cholesterol—The remaining 5 ml. of the acetone solution were transferred to a 50 ml. Erlenmeyer flask and evaporated to dryness. The lipid was redissolved in 5 ml. of petroleum ether and 5 ml. of the digitonin solution were added. From this point it was treated exactly as total cholesterol.

Oxidation—The method of oxidation of the fatty acids is that of Bloor (5). Cholesterol digitonide was oxidized similarly.

Titration with 0.01 N Sodium Hydroxide—This procedure is a modified Stoddard and Drury technique (6). A solution containing about 2 mg. of the fatty acid to be titrated is evaporated to dryness in a 22 × 75 mm. weighing bottle. A wire basket was prepared that would just fit into an ordinary laboratory water bath and copper wire drawn through it at right angles so as to make compartments for the weighing bottles, which can thus be heated in the water bath without danger of tipping. A few grains of clean silica sand are added to prevent bumping. 10 ml. of 95 per cent alcohol are added and boiled for 1 minute. This heating must be done on an electric hot-plate, as the products of a flame increase the acidity of the solution considerably. The weighing bottles are stoppered and cooled by placing the wire basket from the hot water into a water bath containing cold water. The solution is titrated immediately as follows:

4 drops of 3 per cent thymol blue in 50 per cent alcohol are added. The bottle is stoppered and the contents mixed by inverting. Freshly prepared 0.01 N sodium hydroxide solution is added dropwise from a microburette. When the solution begins to turn blue, the base is added with extreme care. The end-point is reached when, after the tube is inverted, there is no longer any yellow color in the solution. One must become accustomed to this point, but once the ability to recognize it is acquired, it can be reproduced with considerable accuracy, and in the opinion of the author, this method is far superior to that with phenolphthalein. The solution is then shaken vigorously for 1 minute, during which process it again becomes slightly yellow, and is finally again

titrated to the end-point. Blanks to correct for the acidity of the alcohol are run simultaneously. The blanks must be boiled in the same manner as the unknown.

Determination of Iodine Numbers—Iodine numbers were determined according to Yasuda (7) without modification.

TABLE I

Oxidation and Titration Factors and Ratios of Fatty Acids

Acid	0.1 N dichromate	0.01 N NaOH	Ratio
	ml.	*ml.*	
Butyric	2.27	1.140	1.99
Caproic	2.70	0.861	3.14
Caprylic	3.06	0.694	4.42
Capric	3 25	0.581	5.60
Lauric	3.40	0.500	6.80
Myristic	3 51	0.438	8.02
Palmitic	3.59	0 390	9.20
Stearic	3.66	0 352	10.40
Oleic	3.62	0 354	10.22
Lignoceric	3.80	0.271	14.02

TABLE II

Lipid Composition of Human Thoracic Duct Lymph

Date of sample	Phospholipid			Cholesterol			Neutral fat	
	Titration of acids	Oxidation of acids	Oxidation of phospholipid	Total	Free	Per cent free	Titration of acids	Oxidation of acids
1935	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*
Feb. 3	68.9	69.7	71.6	30.0	8.6	22.3	318	315
" 6	68.5	65.0	71.6		8.6		300	
" 22	84.8	87.8	86.4	31.4	12.3	28.1	344	328
" 23	74.3	62.4	63.2	23.2	5.5	19.0	337	(286)*

* This figure is probably too low.

Determination of Mean Molecular Weights of Fatty Acids—The object of both oxidizing and titrating the fatty acids is to determine their mean molecular weights. The number of ml. of 0.01 N potassium dichromate required to oxidize 1 mg. of a fatty acid, divided by the number of ml. of 0.01 N sodium hydroxide required to titrate it, is a ratio peculiar to each fatty acid. By determining this ratio of a mixture of fatty acids experimentally, its mean molecular weight can be interpolated, or one may make a graph plotting molecular weights against their ratios. The oxidation and titration factors and the ratios of the more important fatty acids are shown in Table I.

These factors and ratios were determined on palmitic acid (m. p. 63°) and lauric acid (Eastman Kodak Company). The variation from the theoretical values was never greater than 3 per cent, the per cent error of an average of several determinations being less than 1 per cent. The lipid composition of human thoracic duct lymph is shown in Table II; the iodine numbers and the ratio of potassium dichromate to sodium hydroxide of the fatty acids of the lymph are given in Table III.

TABLE III

Iodine Number and Potassium Dichromate to Sodium Hydroxide Ratio of Fatty Acids of Lymph

Date of sample	Iodine No.		Ratio	
	Phospholipid fatty acids	Neutral fat fatty acids	Phospholipid fatty acids	Neutral fat fatty acids
1935				
Feb. 3	95.2	74.5	10.3	10.1
" 22	84 2	69.5	11.2	10.9

DISCUSSION

A survey of the literature reveals very few analyses of human thoracic duct lymph and these were made long before the modern methods of analysis were developed. However, they are given in Table IV for comparison.

Patton (Table IV) called attention to the fact that the amount of magnesium phosphate he weighed in making the determination of phospholipid was so small that his error may have been considerable.

In other animals the value given for phospholipid is much higher and for cholesterol much lower (11–15).

It will be noted the values found in the present analysis agree very well with those of Hoppe-Seyler (Table IV) in the case of phospholipid, but are much lower than his in the case of cholesterol. It must be remembered, however, that the older analysis included all non-saponifiable matter, as determined gravimetrically, and that the present patient had been on a rigid low fat diet for considerable time before the lymph used in this analysis was aspirated.

With the exception of the values as determined by the oxidation of the acids of February 6, and by the titration of the acids of February 23 the greatest difference in the phospholipid values by the three methods of determination is 4.3 per cent. Exclusive of the two discrepancies mentioned, the mean variations of the oxidative and titrative values from the oxidative values of the complete phospholipids are 1.5 and 3.3 per cent respectively. This would indicate that the phospholipids are completely saponified, a conclusion in agreement with Boyd (1) but not with Man and Gildea (16).

TABLE IV

Analyses of Human Thoracic Duct Lymph Recorded in the Literature

Investigator	Phospholipid	Cholesterol	Iodine No.
	mg. per cent	*mg. per cent*	
Patton (8)	36	60	
Hamill (9)	56	70	66.2 (Total fat)
Hoppe-Seyler (10)	75, 88, 83	113, 132	

The large discrepancy of the value of the phospholipid in the sample taken on February 23 as determined by titration (17.6 per cent) might be explained by the presence of contaminating acid from the acidified saponification mixture.

The iodine numbers were calculated on the basis of the quantities as determined by oxidation.

It is interesting to note that the ratio of free to bound cholesterol is similar to that reported by Sperry (17) in the analysis of blood.

By comparing the potassium dichromate to sodium hydroxide ratios (Table I) with those in Table III it is evident that the acids of both the phospholipid and neutral fat have a mean molecular weight very close to stearic and oleic acids, the iodine number suggesting the latter.

To test the method of extraction, the alcohol-lymph layer, after the extraction, was transferred to 200 ml. of 3:1 alcohol-ether solution, filtered, and washed. The filtrate was evaporated to dryness under reduced pressure. The white material remaining was washed with petroleum ether and the washings were added to the original extract.

In another instance lymph was extracted by pipetting into 20 volumes of 3:1 alcohol-ether, boiled, filtered, washed, and the filtrate evaporated to dryness under reduced pressure. The residue was extracted with petroleum ether and the fat weighed.

The results of these analyses gave results no higher than those obtained by the modified Roese-Gottlieb method described.

SUMMARY

1. A new method has been developed for the quantitative partition of the lipids in lymph.

2. A method has been proposed for the microdetermination of the mean molecular weights of fatty acids.

3. The values of phospholipid, total and free cholesterol, triglycerides, and total lipid have been determined for human thoracic duct lymph from a patient on a rigid low fat diet.

4. The iodine numbers and mean molecular weights of the fatty acids of the phospholipid and neutral fats were determined.

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BIBLIOGRAPHY

1. Boyd, E. M., *J. Biol. Chem.*, **101**, 323 (1933).
2. Strauss, A., *J. Thoracic Surg.*, **5**, 539 (1936).
3. Kroeker, E. K., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, **57**, 354 (1935).
4. Boyd, E. M., *J. Biol. Chem.*, **91**, 1 (1931).
5. Bloor, W. R., *J. Biol. Chem.*, **77**, 53 (1928).
6. Stoddard, J. L., and Drury, P. E., *J. Biol. Chem.*, **84**, 741 (1929).
7. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
8. Patton, P. N., *J. Physiol.*, **11**, 109 (1890).

9. Hamill, J. N., *J. Physiol.*, **35**, 151 (1905).
10. Hoppe-Seyler, F., Physiologische Chemie, Berlin, 596 (1877).
11. Rony, H. R., Mortimer, B., and Ivy, A. C., *J. Biol. Chem.*, **96**, 737 (1932).
12. Eckstein, H. C., *J. Biol. Chem.*, **62**, 737 (1924–25).
13. Eckstein, H. C., *Proc. Soc. Exp. Biol. and Med.*, **20**, 74 (1922).
14. Mortimer, B., and Tischer, H. O., *Proc. Soc. Exp. Biol. and Med.*, **31**, 552 (1934).
15. Sullman, H., and Wilbrandt, W., *Biochem. Z.*, **270**, 52 (1934).
16. Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, **99**, 43 (1932–33).
17. Sperry, W. M., *J. Biol. Chem.*, **114**, 125 (1936).

FURTHER STUDIES ON THE ANTIHEMORRHAGIC VITAMIN

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The antihemorrhagic vitamin has previously been obtained in concentrates of high potency (1, 2). The greater portion of the work done on this vitamin has been reviewed (3). It is the purpose of the present report principally to describe in detail further improvements in the purification of the antihemorrhagic vitamin (K) by which it has now been obtained in a crystalline fraction.

Methods and Results

The procedure used for concentration of the antihemorrhagic vitamin (1) has been modified to make it more suitable for the preparation of larger quantities. The vitamin is extracted from commercially dehydrated alfalfa as before. The solvent, a refined hydrocarbon solvent consisting mostly of hexane and having a boiling range of 60–75°, is removed from the extract under reduced pressure. The residue containing the vitamin is then extracted three times with boiling absolute methyl alcohol. Material not dissolved is discarded. The methyl alcohol solution is concentrated to 1 cc. of solution for each 20 gm. of dried alfalfa and stored in large glass centrifuge bottles at 0° for several days. Solids are centrifuged down and the liquid poured off.

The solids are then redissolved in warm absolute methyl alcohol, cooled, and centrifuged as before. The combined filtrates are further concentrated, cooled, and centrifuged until the extract has been worked down to a volume representing about 50 gm. of alfalfa for each cc. of solution. The vitamin is highly soluble in absolute methyl alcohol and advantage may be taken of this property to precipitate a large quantity of inert material. It is necessary to treat such inert material by repeated solutions and chilling in order to minimize losses of the vitamin to a negligible point.

To the concentrated methyl alcohol solutions are added an equal volume of hydrocarbon solvent already referred to and several volumes of water. After standing and separation of layers, the aqueous alcohol layer is drawn off and discarded. The hydrocarbon layer is washed several times more with hot water and then filtered through common filter paper.

A small quantity of solution in a test-tube is treated with Lloyd's reagent until the coloration is reduced to a faint green, the quantity of reagent being noted. One-half of this proportion of Lloyd's reagent is then added slowly with stirring to the bulk of the solution. The adsorbent is removed by filtering or centrifuging. It is then washed once with solvent.

Inert materials in solution are reduced by the above procedure to a point where high vacuum distillation can be readily employed. Such distillation applied at an early stage in the process is very useful in separating the vitamin from remaining pigments and a comparatively large quantity of other substances.

A new type of "molecular" distillation vessel is employed. It consists of a glass tube about 25 mm. in diameter and 40 cm. in length. One end is sealed and rounded; the other is fitted with a female ground glass joint (about 11 mm. in diameter). By means of two circular constrictions indented about 4 mm., the tube is divided into three equal compartments. The compartment at the closed end of the tube will be referred to as *A*, that in the middle as *B*, and that adjacent to the ground glass joint as *C*. On the exterior of *A* is mounted a cylindrical heating element consisting of a close fitting brass tube wound with asbestos, then resistance wire, and finally asbestos. This heating unit is plugged with asbestos at the closed end of *A* and projects past the constriction for about 2 cm. over *B*. *B* is equipped with a similar heating element which projects about 2 cm. over *C*. By means of temperature-recording devices the amount of resistance wire and of external resistance in series can be adjusted until a temperature of about 160° is maintained in *A* and 65° in *B*.

A suitable quantity of concentrate is placed in *A* and the solvent removed by a current of gas and finally by warming under reduced pressure (about 0.01 mm. of mercury). The tube is mounted horizontally and connected to vacuum pumps capable of reducing the pressure to 10^{-5} mm. of mercury or less.

Within a few minutes after pressure has been suitably reduced and heat applied, a colorless oily distillate can be observed in *C*, which is merely air-cooled. By temporarily moving the 160° and 65° heating units, the progress of the distillation into *B* can be watched. The vitamin is found in *B* as an orange, oily fraction volatile at 160° but not at 65°. The 65° distillate (in *C*) contains no detectable quantity of the vitamin (Table I). Periodically, the still is rotated one-half turn while the distillation is proceeding in order to expose a new surface for evaporation. The vitamin fraction from *B* can be further purified by redistillation with a temperature of about 130° in *A*.

This still is the most efficient type we have used. It provides two stages of distillation, prevents mixing of the fractions, and permits ready renewal of the distillation surface, thus affording the maximum possibility for the various substances to reach the proper compartments. A distillation may be completed within 2 hours. The distillates are conveniently removed by cotton wads, and finally by small quantities of solvent.

The fraction obtained in *B* contains sterols, most of which may be removed by chilling in methyl alcohol solution at 0° and centrifuging in the presence of ice. The remaining sterols may be removed by the digitonin procedure.

The sterol-free methyl alcohol solution is next placed in a narrow, conical bottom centrifuge tube mounted in a centrifuge cup having a diameter several times that of the tube. The space between the tube and cup is packed with solid carbon dioxide. After 10 or 15 minutes of cooling, the tube is centrifuged. A precipitate settles firmly in the bottom of the tube. The supernatant liquid is poured off and preserved. The precipitate, which contains the vitamin, is redissolved in a few cc. of methyl alcohol and again chilled out by the same procedure. The clear liquid is again poured off and added to the first. The recrystallization is repeated at least twice more. The process will yield, finally, a colorless solid which liquefies in the presence of adhering solvent at 0°. The supernatant liquid fractions can be concentrated and put through the same procedure to yield a second crop of the vitamin-rich fraction.

The solvent-free vitamin fraction appears microscopically at room temperature as a waxy solid or an extremely viscous liquid. The melting point of the vitamin is probably below ordinary room temperatures.

It was mentioned in a former paper (4) that the oily, pigmented concentrate gave evidence of an indole grouping and of a small content of nitrogen. It is now possible to announce that these results were due to the presence of a substance properly belonging to the 65° distillate but which could not be completely removed from the vitamin fraction without many redistillations in the single stage type of still formerly employed. The improved type of still already described separates the indole fraction very efficiently. This fat-soluble substance giving a distinct indole color reaction should be, in itself, of considerable interest but, since it is not the vitamin we are concerned with, we have devoted little attention to it. It may be stated, however, that, as criteria of purity, tests for the indole substance, nitrogen (micro-Kjeldahl),

TABLE I

Assays for Antihemorrhagic Vitamin

Supplement	Level per kilo of diet	No. of chicks	Age of chicks	Average blood-clotting time
	mg.		*days*	*min.*
Preventive assay				
None		5	7	>30
65° distillate	40	5	7	>30
Crystalline fraction	2	5	7	7.9
" "	4	5	7	5.1
Supernatant "	12	6	7	>30
" "	35	5	7	3.6
	gm.			
None		6	7	>30
Dried alfalfa	5	6	7	2.9
" " sun-bleached	5	7	7	>16
" carrot roots	50	5	7	>30
" " tops	30	6	7	2.7
	mg.			
Curative assay				
None		4	14	>30
Crystalline fraction	4.5	3	14	6.7
" "	6.4	3	14	3.6

and sterols (Liebermann-Burchard) should be negative. On the other hand, the test for a benzene nucleus (4) was found to be particularly strong, indicating that such a structure is contained in the vitamin.

Results of biological assay on various fractions and preparations of the antihemorrhagic vitamin are reported in Table I. The assay procedure has already been described in detail (5). Curative assays when made were performed with 7 day-old chicks whose blood-clotting times were greater than 30 minutes.

DISCUSSION

The results given in Table I show that the vitamin was most highly concentrated in the crystalline fraction obtained by the solid carbon dioxide chilling procedure. Some vitamin remained in the supernatant liquid fraction but it is evident that most of the material in this fraction was inert. Lack of potency in the fraction distilling at 65° agrees with a previous report (1).

The isolation of the antihemorrhagic vitamin for the first time in a colorless crystalline fraction has been described in a preliminary note (6). All previous potent fractions have been in the form of pigmented, viscous oils. This crystalline fraction is at least 8 times as active as the fraction remaining in solution (Table I). The final attainment of a colorless concentrate is in agreement with other evidence (4) that the vitamin is colorless.

Treatment of a sample of dried alfalfa by sunlight sufficient to cause noticeable bleaching was destructive to the vitamin, in agreement with results of a similar experiment conducted with a purer form (4). As raw material for the isolation of the vitamin, artificially dried alfalfa products are likely to be superior to field-dried products that have been exposed to sunlight.

It has been reported that carrot roots containing no green substance failed to prevent hemorrhagic symptoms (7). This is confirmed by the results from feeding dried carrot roots (Table I). On the other hand, the dried carrot tops were an effective source of the vitamin. The antihemorrhagic vitamin distribution evidently differs radically from that of provitamin A (carotene) and appears to be confined entirely to the photosynthetic portion of the plant. It is generally true that the richest sources of the vitamin are the green leafy portions of plants.

The requirement of the chick for the antihemorrhagic vitamin, as indicated by the data in Table I, is higher than that reported previously (1). The former report was based upon the presence of external hemorrhagic symptoms which under our experimental conditions are very readily induced. It is possible that the chicks showing no hemorrhagic symptoms may, however, have had somewhat prolonged clotting times and that their diets may have been suboptimal in content of the antihemorrhagic vitamin. The reserve of the vitamin in the newly hatched chick may also vary from group to group and thus exert some influence on the level apparently required in the diet.

SUMMARY

1. An improved process for purification of the antihemorrhagic vitamin is described.

2. The vitamin has been obtained in the form of a colorless crystalline fraction of low melting point. It contains one or more benzene rings.

BIBLIOGRAPHY

1. Almquist, H. J., *J. Biol. Chem.*, **114,** 241 (1936); **115,** 589 (1936).
2. Dam, H., and Lewis, L., *Biochem. J.*, **31,** 17 (1937).
3. Almquist, H. J., *Poultry Sc.*, **16,** 166 (1937).
4. Almquist, H. J., *J. Biol. Chem.*, **117,** 517 (1937).
5. Almquist, H. J., and Stokstad, E. L. R., *J. Nutrition,* in press.
6. Almquist, H. J., *Nature,* **140,** 25 (1937).
7. Almquist, H. J., and Stokstad, E. L. R., *J. Nutrition,* **13,** 339 (1937).

THE INFRA-RED ABSORPTION SPECTRA OF THE STEREOISOMERS OF CYSTINE

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The isolation of the *l*, *d*, *dl*, and meso forms of cystine has been described by du Vigneaud and his coworkers (1–3). Chemical methods and also microscopical studies of the crystals have been used (3) to characterize the meso or internally compensated form of cystine. Solubility data (4) have provided very strong evidence that the *dl* form of cystine, obtained in the manner described (2), is a racemic compound and not a racemic mixture.

It is very well known that the selective absorption of substances in the infra-red region of the spectrum originates primarily in the vibrations of atoms and groups of atoms within molecules. In the case of solid crystalline substances, such as the cystines, the frequencies of vibration within the individual molecules are influenced to some extent by the neighboring molecules; hence the spectra are characterized by the crystalline structure as well as by the type of molecule.

The present study of the infra-red absorption spectra of the stereoisomers of cystine demonstrates some of the molecular and structural differences of these compounds by an entirely independent method.

The spectra were observed with the vacuum, self-recording infra-red spectrometer described by Randall and Strong (5), with the periodic amplifier designed by Firestone (6). In this spectrometer a 60° potassium bromide prism is mounted in a combination Wadsworth-Littrow system; the spectral range is from 1 to 25μ. The customary Nernst glower furnished the radiation.

The method of obtaining the absorption spectra of the various forms of cystine was to place a thin layer of the finely powdered material in question in the path of the radiation entering the spectrometer. The layers were prepared in each instance by stirring up the powder in absolute alcohol and allowing it to settle out on a potassium bromide plate. The alcohol was then removed by evaporation, leaving a fairly uniform layer which contained about 1 mg. of powder per sq. cm. Layers of the different forms of cystine were prepared under conditions as nearly identical as possible. It is noteworthy that an amount as small as 5 mg. is sufficient for observation of the spectrum.

Highly purified samples of the various isomers of cystine examined in this work (1–3) were very kindly supplied by Dr. Vincent du Vigneaud, of George Washington University, whom the writer takes this opportunity to thank. The *l*-cystine from protein and from cystinuric urine (7) was made available through the kindness of Dr. H. B. Lewis.

The curves appearing in Fig. 1 represent the intensity of the radiation transmitted by the various powder layers as a function of the wave-length. Except for the general scattering of the powder particles the contours of these curves follow the emission curve of the Nernst glower; the numerous minima are due to the characteristic absorption of the cystines placed in the path of the radiation. The spectrum has been divided into several wave-length intervals owing to the necessity of changing the slit widths or the sensitivity of the instrument. In each of these intervals, however, the curves have been obtained under identical conditions. Owing to the small dispersion of the prism in the region from 1 to 6μ and the consequent lack of significant detail, this interval has been omitted in Fig. 1.

Lists of the wave-lengths and corresponding wave numbers of the absorption maxima (minima in the curves of Fig. 1) of the different forms of cystine are contained in Table I. In the columns designated intensity are approximate percentages of the unscattered radiation absorbed at the maxima.

The spectra of *l*- and *d*-cystine (Curves 1 and 2 respectively, Fig. 1) are identical within the limits of experimental error. There is indeed no alternative to this result if the molecules and the crystals, respectively, of these two isomers are mirror images of each other, since all factors influencing the vibration frequencies are exactly equivalent in the two cases.

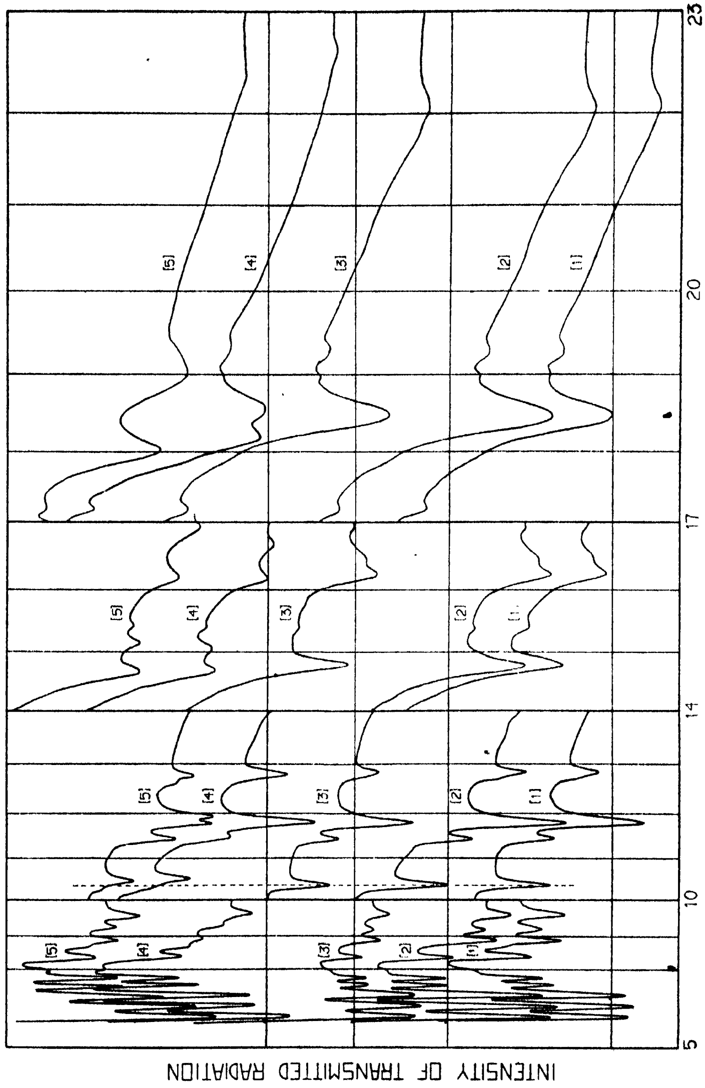

FIG. 1. Infra-red spectra of powder layers of the cystines. *l*-Cystine, Curve 1; *d*-cystine, Curve 2; mechanical mixture of *l*- and *d*-cystines, Curve 3; *dl*-cystine, Curve 4; mesocystine, Curve 5.

Measurements by Heintz (8) on the spectrum of *l*-cystine in the region from 1 to 9μ have apparently been made with a somewhat lower resolving power than here applied, but the agreement is as good as this experimental difference will permit.

TABLE I

Wave-Lengths, Wave Numbers, and Intensities of Infra-Red Spectra of Isomeric Forms of Cystine

The spectra of the *l* and *d* forms of cystine are identical.

l-Cystine or *d*-cystine			*dl*-Cystine (compound)			Mesocystine		
Wave-length	Wave No.	Intensity	Wave-length	Wave No.	Intensity	Wave-length	Wave No.	Intensity
μ	$cm.^{-1}$	*per cent*	μ	$cm.^{-1}$	*per cent*	μ	$cm.^{-1}$	*per cent*
3.30	3030	90	3.36	2980	90	3.38	2960	90
3.79	2640	10	4.80	2083	10	6.28	1592	90
4.71	2123	10	6.27	1595	90	6.69	1495	80
6.27	1595	90	6.72	1488	80	7.06	1416	70
6.71	1490	90	7.10	1408	70	7.43	1346	50
7.11	1406	80	7.47	1339	50	7.73	1294	50
7.47	1339	60	7.71	1297	40	7.98	1253	10
7.70	1299	50	8.00	1250	10	8.38	1193	30
7.93	1261	10	8.40	1190	30	8.70	1149	10
8.36	1196	40	8.70	1149	10	8.88	1126	30
8.86	1129	40	8.92	1121	20	9.28	1078	20
9.17	1091	30	9.27	1079	10	9.57	1045	30
9.56	1046	40	9.62	1040	30	10.48	954	20
10.35	966	40	10.52	951	20	11.45	873	40
11.44	874	30	11.47	872	20	11.77	850	40
11.81	847	80	11.82	846	80	11.95	837	40
12.85	778	30	12.80	781	50	12.63	792	10
14.79	676	60	14.68	681	30	12.77	783	30
15.38	650	10	15 06	664	20	14.68	681	40
16.24	616	40	15.40	649	10	15.15	660	20
16.50	606	20	16.18	618	30	15.43	648	10
17.18	582	10	16.69	599	20	16.17	618	20
18.47	541	70	17.20	581	10	16.92	591	20
19.27	519	10	18.18	550	40	17.21	581	10
21.68	461	30	18.57	538	40	18.00	556	40
22.06	453	50	19.29	518	10	19.00	526	40
			22.58	443	30	22.34	448	30

Curve 3 (Fig. 1) portrays the spectrum of a mechanical mixture of equal amounts of the dry powders of *l*- and *d*-cystine. It is, of course, identical with the spectra of *l*- and *d*-cystine taken separately but serves for comparison with Curve 4.

It is obvious at a glance, however, that the spectrum of *dl*-cystine (Curve 4, Fig. 1), obtained by crystallization from solution (2), possesses many significant differences from that of the mechanical mixture. Several of the absorption frequencies (minima in the curve) are seen to be shifted from the corresponding frequencies of the active isomers (or of the mechanical mixture). In at least two instances (14.79μ and 18.47μ), single absorption frequencies of the active isomers become pairs in the *dl* compound. These spectral differences reveal unmistakably that the *dl*-cystine has an individual crystalline form, or, in other words, is a compound and not a mere mixture of *d* and *l* crystals.

Mesocystine possesses a spectrum (Curve 5, Fig. 1) even more divergent from the active isomers than *dl*-cystine. As we are here dealing with a type of difference in molecular structure which must have a decided influence on the internal force constants, the spectrum can be expected to show greater differences than would be the case for a change in crystal structure only.

As a matter of further interest, the spectra of samples of *l*-cystine obtained from hydrolysis of protein and from cystinuric urine (7) proved to be identical.

SUMMARY

The infra-red absorption spectra of the *l*, *d*, *dl*, and meso forms of cystine have been recorded.

Comparison of the spectra have shown that *dl*-cystine as obtained by crystallization from solution is a compound and not a mixture.

The spectrum of mesocystine showed a marked divergence from that of the optically active cystines, as expected from its difference in molecular structure.

Samples of *l*-cystine from protein and from cystinuric urine had identical spectra.

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BIBLIOGRAPHY

1. Hollander, L., and du Vigneaud, V., *J. Biol. Chem.*, **94**, 243 (1931–32).
2. du Vigneaud, V., Dorfman, R., and Loring, H. S., *J. Biol. Chem.*, **98**, 577 (1932).
3. Loring, H. S., and du Vigneaud, V., *J. Biol. Chem.*, **102**, 287 (1933).
4. Loring, H. S., and du Vigneaud, V., *J. Biol. Chem.*, **107**, 267 (1934).
5. Randall, H. M., and Strong, J., *Rev. Scient. Instruments*, **2**, 585 (1931).
6. Firestone, F. A., *Rev. Scient. Instruments*, **3**, 163 (1932).
7. Brown, B. H., and Lewis, H. B., *Proc. Soc. Exp. Biol. and Med.*, **32**, 1100 (1934–35).
8. Heintz, E., *Compt. rend. Acad.*, **201**, 1478 (1935).

THE EFFECT OF CHOLINE ON THE LIPID METABOLISM OF BLOOD AND LIVER IN THE COMPLETELY DEPANCREATIZED DOG MAINTAINED WITH INSULIN*

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Previous studies from this laboratory (1, 2) have shown that pancreas contains two factors active in the lipid metabolism of the depancreatized dog maintained with insulin: (1) a heat-labile *blood* factor, the ingestion of which causes a rise above normal in all blood lipid constituents, in particular cholesterol; this factor is partially or completely destroyed when pancreas is subjected to autoclaving at 20 pounds steam pressure for 30 minutes; (2) a heat-stable *liver* factor, the administration of which prevents the deposition of fat in the liver; this factor is not destroyed by the above heat treatment.

The curative action of lecithin on the fatty liver of the depancreatized dog receiving insulin was discovered by Hershey (3). A later study by Best and his coworkers (4) showed that its active constituent is choline, and these workers ascribe the action of pancreas on the *liver* to its choline content. Dragstedt and his coworkers (5), however, claim to have ruled out choline as the active factor in the pancreas. For 2 to 4 weeks they fed depancreatized dogs choline in amounts up to 700 mg. daily (the latter amount being that contained in approximately 250 gm. of pancreas) and this treatment failed to cure fatty livers. But it was found in this laboratory (2) that raw pancreas, even when fed in amounts

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as great as 250 gm. daily, does not empty the liver of abnormal amounts of fat in as short a period as 3 weeks. In three dogs that had received 250 gm. of the raw glandular tissue for 4 to 5.5 weeks after fatty livers had been established, 29, 27, and 23 per cent fatty acids were still present in mixed samples of the liver. The curative action of the raw glandular tissue is a slow process, and a period exceeding 5.5 weeks is required to empty the liver of abnormal amounts of fat once it has been deposited. As further evidence against the view that choline is the active factor in pancreas, Dragstedt *et al.* (5) fed liver and brain, tissues rich in lecithin, and reported failure of these to cure fatty livers. But a curative action is not to be expected from these two tissues despite their high phospholipid content. In the first place, Blatherwick *et al.* (6) demonstrated several years ago that fatty livers are produced in rats by the feeding of liver. This, indeed, has been amply confirmed by Beeston and Wilkinson (7). Furthermore, while brain is rich in phospholipids, it also contains cholesterol, the feeding of which to rats has been shown by many workers to produce fatty livers. An example of the type of reaction to be expected from feeding such tissues is provided by the work of Okey and her coworkers (8), in which it was shown that egg yolk, though exceptionally rich in phospholipids, nevertheless (owing to its cholesterol content) results in fatty livers when fed to rats. While no attempt is here made to associate the pancreatic *liver factor* with choline, it yet seems necessary to point out that a more rigid proof than that offered by Dragstedt *et al.*[1] must be provided before their dissimilarity can be accepted.

The relation of choline to the lipid changes in the *blood* of completely depancreatized dogs maintained with insulin has not hitherto been investigated. The fact that the pancreatic *blood factor* is heat-labile suggests, however, that this factor is not choline.

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[1] It has previously been pointed out (9) that the method of fat estimation employed by Dragstedt *et al.* is open to question. Moreover, in their choline studies they record four dogs, two of which died in 4 to 5 weeks after pancreatectomy despite the addition of choline to the diet. From the fact that long survival of a large number of completely depancreatized dogs has now been demonstrated in this laboratory without the aid of choline supplements, it is difficult to accept the data offered by these workers.

EXPERIMENTAL

The preparation and maintenance of the completely depancreatized dogs used in this study have been previously described (2). Following pancreatectomy each dog received twice daily, at 8 a.m. and 4 p.m., a mixture containing 280 gm. of lean meat, 50 gm. of sucrose, and 7 gm. of bone ash. Vitamin supplements (A and D as cod liver oil;[2] the B complex in the form of a concentrate[3] obtained from rice bran) were added to the diet mixture twice a week. Each animal received 16 units of insulin daily, 8 units at each time of feeding. When it was desired to administer choline chloride, this was mixed with the diet a few minutes before the meal was served.

Blood and liver were taken for analyses between 8 and 9 a.m.; the dogs had received their last injection of insulin and their last meal at 4 p.m. of the previous day. This state of the animal in which it has been deprived of both food and insulin for 16 hours is here referred to as the postabsorptive state.

The liver was removed after the animal had been anesthetized with sodium amytal. A mixed sample of the whole liver was used for lipid estimation. The method of sampling the liver and the procedures employed for lipid determinations of liver and blood have been previously described (11).

Liver Lipids

Nine completely depancreatized dogs maintained with insulin received choline supplements at various intervals following excision of the gland (Table I). In a single animal (Dog D-27) the daily administration of 2.5 gm. of choline chloride was begun immediately after pancreatectomy[4] and was continued for 11 weeks. At

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[2] The standardized cod liver oil used in this study was kindly furnished by Mead Johnson and Company.

[3] The rice bran concentrate was kindly furnished by Vitab Products, Inc., Emeryville, California. This concentrate contained 55 international units of vitamin B_1 and 10 modified Bourquin-Sherman units of vitamin G (flavin) per cc. The same concentrate has also been shown to be a good source of both rat and chick antidermatitis factors (10).

[4] Although animals may suffer a slight impairment in appetite for a short interval immediately following the operation, Dog D-27 showed no such effect, vigorously ingesting all diet mixtures including the choline supplements fed after pancreatectomy, which were begun on the day following the operation.

TABLE

Curative and Preventive Action of Choline upon Fatty Livers of Completely Depancreatized Dogs Maintained with Insulin

Dog No.	Weight	Period depancreatized	Period receiving choline	Liver		Cholesterol				Total lipid	Total fatty acids	Phospholipid	Residual fatty acids
				Weight	Per cent of body weight	Total	Free	Ester					
	kg.	*wks.*	*wks.*	*gm.*		*per cent*	*per cent*	*per cent*	*per cent of total*	*per cent*	*per cent*	*per cent*	*per cent*
D-27	10.5	11	11*	320	3.0	0.26	0.24	0.02	8	3.84	2.50	2.35	2.26
D-15	7.0	10	Last 3†	375	5.4	0.25	0.21	0.04	16	22.2	19.6	2.14	20.7
D-21	6.3	17	" 6‡	545	8.7	0.36	0.16	0.20	56	25.7	23.6	1.61	24.5
D-23	6.8	18	" 6‡	315	4.6	0.52	0.19	0.33	63	14.2	12.4	1.93	12.7
D-48	8.7	26.5	" 11.5‡	490	5.6	0.43	0.25	0.18	42	7.07	5.66	1.40	6.00
D-49	7.2	26	" 11.5‡	365	5.1	0.30	0.18	0.12	40	7.00	5.70	1.49	5.91
D-77	8.0	36.5	" 15‡	365	4.6					6.92	5.35		
D-82	8.5	35	" 15‡	395	4.6						11.0		
D-81	5.8	35	" 15‡	400	6.9	0.35	0 25	0.10	29	15.1	13.3	1.83	13.8

* 2.5 gm. of choline chloride daily.

† 3.0 gm. of choline chloride daily.

‡ 2.0 gm. of choline chloride daily.

the end of this time the animal was sacrificed for lipid analysis of the liver. The liver showed no enlargement. Total fatty acids were present to the extent of 2.5 per cent. In a large series of normal animals maintained in this laboratory the livers contained up to 2.7 per cent fatty acids. The other lipid constituents in the liver of Dog D-27, namely cholesterol and phospholipids, were also present in normal amounts.

In eight dogs the administration of choline chloride was begun at various intervals after pancreatectomy. These may be grouped as follows:

1. The administration of 3 gm. of choline chloride daily for 3 weeks, begun at an interval of 7 weeks after pancreatectomy, failed to empty the liver of its fat.
2. In two dogs the feeding of 2 gm. daily was begun 11 and 12 weeks after pancreatectomy and continued for 6 weeks. Despite this treatment 25.7 and 14.2 per cent of total lipids were still present in the livers.
3. The livers of Dogs D-48 and D-49 were analyzed 26.5 and 26 weeks after pancreatectomy, during the last 11.5 weeks of which they received daily 2 gm. of choline chloride. The fatty acid content of these livers was reduced to 5.7 per cent. In a study of the time of onset of fatty livers in depancreatized dogs, fatty acids in excess of 14 per cent were found at intervals of 20 to 36 weeks after pancreatectomy (2).
4. Three dogs received 2 gm. of choline chloride daily for the last 15 weeks of their 35 to 36.5 week period of maintenance after pancreatectomy. Although the lipid content of their livers was reduced below that found at this time interval after pancreatectomy in animals not receiving choline supplements (2), it should be noted that the lipid content of the livers of Dogs D-77, D-81, and D-82 was still above normal. The lowest lipid value was found in the liver of Dog D-77, in which 5.4 per cent of fatty acids was present.

Blood Lipids

Feeding of Choline at Various Intervals after Pancreatectomy[5]—In a single dog (No. D-27) the administration of 2.5 gm. of choline chloride was begun the day following pancreatectomy and continued daily for 11 weeks (Table II). The preoperative levels for free and ester cholesterol were respectively 127 and 31 mg. per cent, whereas 11 weeks after choline administration these two constituents were present to the extent of 100 and 47 mg. per 100 cc. of blood. The preoperative and final blood samples did not differ significantly in their phospholipid or total fatty acid contents.

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[5] A progress report containing other data has appeared (12).

TABLE II

Effect of Ingestion of Choline upon Postabsorptive Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin

Dog No.	Weight	Period after pancreatectomy	Period receiving choline	Cholesterol				Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
				Total	Free	Ester					
	kg.	wks.	wks.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent of total	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
D-27	8.8	Preoperative		158	127	31	20	360	301	518	127
	8.8	5	5*	159	114	45	28	429	348	588	163
	9.7	8.5	8.5	159	123	36	23	410	392	569	121
	10.5	11	11	147	100	47	32	335	335	482	77
D-15	8.5	Preoperative		150	118	32	21				
	7.5	7	0	105	104	1	1	284	276	389	109
	7.0	10	3†	153	125	28	18	399	358	552	139
D-21	7.7	Preoperative		131	103	28	21	328	316	459	96
	6.0	11	0	132	133	0	0	347	282	479	158
	6.3	17	6‡	142	124	18	13	386	284	528	183
D-48	10.1	Preoperative		155	108	47	30	361	334	516	103
			0	110	110	0	0	234	227	344	82
			11.5‡	166	131	35	21	428	405	594	130
D-49	6.9	14.5	0	123	100	23	19	250	271	373	62
	7.2	26	11.5‡	153	131	22	14	450	393	603	171
D-81	4.9	20	0	129	106	23	18	250	242	379	71
	6.0	31	11‡	142	110	32	23				
	5.8	35	15	148	111	37	25	368	378	478	88
D-82	7.7	20	0	102	91	11	11	243	216	345	92
	8.5	31	11‡	123	112	11	9	420	373	563	162
	8.5	35	15	135	107	28	20				

* 2.5 gm. of choline chloride daily.

† 3.0 gm. of choline chloride daily.

‡ 2.0 gm. of choline chloride daily.

It was previously shown that a lowered lipid level can be produced after pancreatectomy by feeding diets containing no raw pancreas (13). In order to observe its effect upon this condition, the administration of choline was begun in six dogs at various intervals after pancreatectomy when a lowered blood lipid level had been established.

In Dogs D-15, D-21, and D-48, the administration of choline restored the blood lipids to the preoperative level. 7 weeks after Dog D-15 was depancreatized, total and ester cholesterol fell from preoperative values of 150 and 32 mg. to 105 and 1 mg. per 100 cc. of blood. The administration of 3 gm. of choline daily was begun at this time and continued for 3 weeks thereafter. At the end of this period the blood of Dog D-15 contained 153 and 28 mg. per cent of total and esterified cholesterol respectively. Similar changes in cholesterol esters occurred in Dogs D-21 and D-48. In these two it may be further observed that, although total fatty acids and total lipids dropped after pancreatectomy, both constituents showed a rise to values close to preoperative as a result of choline feeding.

TABLE III

Effect of Substitution of Choline for Raw Pancreas upon Blood Lipids of Completely Depancreatized Dog (No. D-55) Maintained with Insulin

Interval since pancreatectomy	Period receiving raw pancreas	Period receiving 2.0 gm. of choline chloride daily	Weight	Cholesterol				Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
				Total	Free	Ester	Ester				
days	*days*	*days*	*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent of total*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Preoperative			11.5	172	119	53	31	430	390	602	129
142	142		11.5	236	136	100	42	448	443	684	79
154		12	11.0	185	124	61	33	474	424	659	145
162		20	10.8	162	112	50	31	448	421	610	129
172		30	10.5	186	128	58	31	541	401	727	221
181	9		11.2	250	143	107	43	625	451	875	245
214	42		11.7	236	134	102	43	618	479	854	223

Although cholesterol esters rose somewhat after choline administration in the three dogs mentioned above, it should be observed that a rise above the normal or preoperative level did not occur. Further evidence of the inability of choline to effect a rise in blood cholesterol above normal is shown in Dogs D-49, D-81, and D-82. In Dog D-49, 20 mg. per cent of cholesterol esters were still present in the blood at an interval of 14.5 weeks after pancreatectomy, and although total cholesterol rose from 123 to 153 mg. per cent as a result of the ingestion of 2 gm. of choline chloride daily for 11.5 weeks, cholesterol esters showed practically no change. The cholesterol levels in the blood of Dogs D-81 and D-82 also rose above the values found at the time choline feeding was begun, but the lipid values attained after 15 weeks of choline ingestion were still within normal range.

Substitution of Choline for Raw Pancreas—Further evidence of the dissimilarity of choline and raw pancreas appears in Table III, which shows the effects of choline upon a high blood lipid level produced by the feeding of raw pancreas. Dog D-55 received raw pancreas for 142 days after pancreatectomy, and by the end of this interval total cholesterol had risen from 172 to 236 mg. per cent, while cholesterol esters had practically doubled, attaining a value of 100 mg. per 100 cc. of blood. 20 days after choline had been substituted for the raw glandular tissue, the blood lipids returned to approximately the preoperative level. It is interesting to note, however, that despite the fact that choline administration was continued for 10 days more, none of the blood lipid constituents fell below the normal level. Raw pancreas was now substituted for choline, and by the end of 9 days total cholesterol had increased by 64 mg. per cent and cholesterol esters by 49 mg.

DISCUSSION

Choline in large amounts influences the deposition of fat in the livers of depancreatized dogs maintained with insulin. But its curative action upon those livers in which a marked fatty infiltration was already present is slow, and a feeding interval longer than 3 weeks is necessary to effect a measurable change even when so large an amount as 0.4 gm. per kilo of body weight is being administered. The extent of the delay in this curative action is well brought out by the fact that 26 per cent of total lipids remained in the liver of a 6.3 kilo dog (Table I) despite the ingestion of 2 gm. of choline chloride daily during the 6 weeks preceding the examination of the liver. Indeed, the feeding of the same amount to dogs weighing between 5.8 and 8.0 kilos for a period of 15 weeks failed to restore the liver lipids to normal levels. A somewhat similar phenomenon was observed in the action of *pancreas* upon the fatty livers of depancreatized dogs (2). While the daily administration of pancreas begun immediately after pancreatectomy readily prevented the deposition of abnormal amounts of fat, its curative action upon livers once they had accumulated large amounts of fat was slow, and a period longer than 6 weeks was required to demonstrate clearly a measurable decrease in liver lipids by means of the ingestion of 250 gm. of the glandular tissue daily.

Significance of Choline Content of Stock Diet—The stock diet employed in the present investigation contained meat, sucrose, bone ash, and vitamin supplements and is obviously not free from choline. Each animal receives approximately 400 mg. of choline daily from this dietary mixture. Yet this amount does not prevent the deposition of large amounts of lipids in the livers of depancreatized dogs. In a study of the time of onset of these fatty changes (2), it was shown that 31 per cent of total lipids may be present in the livers of animals that have received this stock diet for 20 weeks after pancreatectomy. Since by far the largest amount of choline in the stock diet is contained in the lecithin of the meat, it is interesting to record here that in a single depancreatized dog (No. D-85) weighing 9 kilos the ingestion of 1120 gm. of meat daily failed to empty the liver of its abnormal amounts of fat (2). This animal was fed the stock diet containing 560 gm. of lean meat daily for the first 20 weeks after pancreatectomy in order to establish a fatty liver. For the next 15 weeks it received 1120 gm. of lean meat in addition to the other dietary constituents. A mixed sample of the whole liver removed at the end of the 15 week period contained 27 per cent of fatty acids. It is estimated that, during the time in which the animal received 1120 gm. of meat, it ingested approximately 800 mg. of choline daily. This apparent inactivity might be explained in various ways. It is by no means unlikely that, in the absence of pancreatic enzymes, the choline combined in the lecithin of the meat is not so readily available to the animal as when free choline is fed.

Relation of Choline to Pancreatic Liver Factor—In view of the activity of choline when fed as the chloride, due consideration must be given to the choline content of the ingested pancreas in any attempt to determine the factor (or factors) in this glandular tissue that influences the lipid content of the liver of depancreatized dogs. In this laboratory it has been found that the daily ingestion of 250 gm. of pancreas prevents the deposition of abnormal amounts of fat in the livers of such animals (2). This amount of pancreas provides about 600 to 700 mg. of choline daily. If choline is the only active factor in the pancreas, it should be possible to show: (1) that the physiological action upon the liver by a given sample of pancreas can be equated with that of the choline which can be isolated from it; (2) that the activity of any pancreatic *fraction*[6] is related to its choline content and that the removal of choline from such a fraction results in loss of activity. Thus far no such evidence has been provided, and in its absence we must suspend judgment as to whether pancreatic action upon the liver is due solely to its choline content or whether pancreas contains a liver factor in addition to the choline.

Relation of Choline to Pancreatic Blood Factor—The present study shows quite clearly that the factor in pancreas that raises the blood lipids of the depancreatized dog above normal is not choline. But it should be noted that, though failing to effect such a rise, choline, when added to the diet in large amounts, nevertheless prevented the fall in blood lipids below normal, which occurs after pancreatectomy in dogs receiving the stock diet of this laboratory. A somewhat similar interrelation between the lipid metabolism of blood and liver was observed in the action of autoclaved pancreas on the depancreatized dog treated with insulin (2). This autoclaved tissue prevents as well as cures fat deposition in the liver, and there is associated with this action the maintenance of a normal blood lipid level. It would seem, therefore, that the fall below normal observed in the blood lipid constituents of depancreatized dogs kept alive with insulin is related to the production of a fatty liver, since choline and autoclaved pancreas, both of which prevent abnormal amounts of fat from being deposited in the liver, also prevent the fall in blood lipids. While, on the one hand, the fall in blood lipids is probably associated with the accumulation of fat in the liver, on the other hand the rise above normal observed after raw pancreas ingestion is not due to the liver's being emptied of its large amount of fat, since a rise in the blood lipids can be produced by raw pancreas immediately after pancreatectomy; *i.e.*, before abnormal amounts of fat have accumulated in the liver.

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[6] Although choline influences the fatty livers of rats fed a high fat, low protein diet as well as those of depancreatized dogs maintained on a low fat, high protein diet, the evidence available at present does not permit the conclusion that the mechanism of production and cure of these two types of fatty livers is the same. Hence conclusions derived from experiments on rats cannot be applied at present to depancreatized dogs.

SUMMARY

1. The preventive and curative actions of choline upon the fatty livers of completely depancreatized dogs maintained with insulin were investigated. While it is shown that choline in large amounts influences the deposition of liver fat, its curative action is slow, and daily feeding for a long time is required to produce measurable effects on livers in which large amounts of fat have accumulated.

2. The significance of choline in the action of pancreas upon the liver lipids of completely depancreatized dogs is discussed.

3. It is shown that choline does not raise the blood lipids in depancreatized dogs above the normal level. The pancreatic blood lipid factor is therefore not choline.

BIBLIOGRAPHY

1. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **112**, 155 (1935–36).
2. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **119**, 435 (1937).
3. Hershey, J. M., *Am. J. Physiol.*, **93**, 675 (1930). Hershey, J. M., and Soskin, S., *Am. J. Physiol.*, **98**, 74 (1931).
4. Best, C. H., Ferguson, G. C., and Hershey, J. M., *J. Physiol.*, **79**, 94 (1933).
5. Van Prohaska, J., Harms, H. P., and Dragstedt, L. R., *Am. J. Physiol.*, **116**, 122 (1936). Van Prohaska, J., Dragstedt, L. R., and Harms, H. P., *Am. J. Physiol.*, **117**, 166 (1936).
6. Blatherwick, N. R., Medlar, E. M., Bradshaw, P. J., Post, A. L., and Sawyer, S. D., *J. Biol. Chem.*, **103**, 93 (1933).
7. Beeston, A. W., and Wilkinson, H., *Biochem. J.*, **30**, 121 (1936).
8. Okey, R., Yokela, E., and Knock, G., *Proc. Soc. Exp. Biol. and Med.*, **31**, 507 (1934); *J. Nutrition*, **11**, 463 (1936).
9. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **119**, 423 (1937).
10. Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, **115**, 557 (1936).
11. Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, **108**, 201 (1935).
12. Chaikoff, I. L., and Kaplan, A., *Proc. Soc. Exp. Biol. and Med.*, **34**, 413 (1936).
13. Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, **106**, 267 (1934).

THE EFFECT OF PYOCYANINE ON THE METABOLISM OF CEREBRAL CORTEX

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The fact that increased oxygen consumption can be induced in living animal cells by certain oxidation-reduction dyes was demonstrated by Harrop and Barron (1928) and Barron and Harrop (1928); according to Barron and Hoffman (1930) it is dependent upon the rate of reduction of the dye within the cell and its oxidation by atmospheric oxygen proceeding more rapidly than normal cellular oxidation processes. After studying the action of methylene blue on slices of mammalian tissue Barron (1930) stated that the dye increased the oxygen consumption of tumors and of those normal tissues having aerobic glycolysis, the effect being roughly proportional to the fermentative power of the tissues. Friedheim (1934) found that pyocyanine was less toxic to tissues than methylene blue and that it could cause an increase in the respiration of tumors together with a decrease in the aerobic glycolysis. Dickens (1936) showed, however, that the decreased aerobic glycolysis was not dependent merely on the increased oxygen consumption, since pyocyanine did not increase the R.Q. and since it also inhibited the anaerobic glycolysis of tumors.

While the action of pyocyanine has been studied in detail on a tissue with high aerobic glycolysis, namely tumor, data have hitherto been lacking on its effect on cerebral cortex, a tissue with low aerobic glycolysis. The work to be described shows that its action on brain cortex differs in certain respects from that on tumor. In concentrations varying from 2×10^{-3} M to 4×10^{-5} M pyocyanine causes an initial increase in the oxygen consumption of slices of rabbit cerebral cortex, and this is followed by an inhibition which is very marked with high concentrations of pyocyanine. The inhibitory action is irreversible. The degree to which oxidation is accelerated in brain cortex by pyocyanine is dependent on the nature of the substrate added, the maximal effect being obtained with glucose. No increased oxidation is obtained with pyocyanine in the absence of added substrate and only a slight effect is observed with low tensions of oxygen. Pyocyanine has a marked action on the glycolytic mechanisms of cerebral cortex. High concentrations of the dye cause increased aerobic glycolysis, the onset of which occurs during the period of respiratory stimulation. All concentrations of the dye tested increase anaerobic glycolysis initially, but the effect is only maintained with the lower concentrations. Pyocyanine has little effect on the respiration of cerebral cortex in the presence of 0.001 M cyanide. With brain cortex treated with 0.1 M KCl, which is known to produce increased respiration and increased aerobic glycolysis (Ashford and Dixon, 1935; Dickens and Greville, 1935) the initial increase of respiration under suitable conditions due to the combined action of 0.1 M KCl and pyocyanine is approximately equal to the sum of their separate effects.

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* Commonwealth Fund Fellow in Biochemistry.

Methods and Reagents

The manometric methods used were those described by Warburg (1926) and Dixon (1934). Slices of cerebral cortex of adult rabbits which had been killed by decapitation were used in all experiments unless otherwise stated. All experiments were repeated at least twice with different animals. In general each experiment lasted 5 hours, the dye being added from the side arm of the Warburg flask after a control period lasting 1 hour. All experiments were conducted with 2 ml. of suspension medium at pH 7.4 and a temperature of 37.5°. The suspension medium used (0.120 M $NaCl$, 0.0024 M KCl, 0.0017 M $CaCl_2$, 0.0008 M $MgCl_2$) was a modification of the Warburg (1923) medium described by Dickens and Greville (1935). Substrate when present was added in a concentration of 0.2 per cent. For the measurement of respiration in phosphate buffer 0.0033 M NaH_2PO_4 and 0.015 M Na_2HPO_4 were added to the above medium, and for measurement of respiration by the original Warburg technique (low bicarbonate concentration, absorption of CO_2 by KOH) 0.0025 M $NaHCO_3$ was added. The medium for the determination of acid production contained 0.025 M $NaHCO_3$; an atmosphere of N_2 containing 5 per cent CO_2 was used for the measurement of anaerobic glycolysis, and 5 per cent CO_2 in O_2 was used for aerobic acid production measurements. For the removal of traces of O_2 from the N_2-CO_2 the gas mixture was passed over copper heated in an electric furnace and the issuing gas was bubbled through water before passing into the Warburg flasks. In the chemical determination of lactic acid the medium containing the slices was made 6 per cent with respect to trichloroacetic acid, filtered, the filtrate treated with copper sulfate and lime, and the solution obtained by centrifugation was used for lactic acid determination by the Wendel (1933) micromodification of the method of Friedemann, Cotonio, and Shaffer (1927). The pyocyanine used, unless otherwise stated, was a sample prepared from *Pseudomonas pyocyanea* and twice precipitated as hydrochloride (Elema and Sanders, 1931). Solutions of the hydrochloride were neutralized before use, and blank experiments were performed to insure that they did not cause a significant gas change on addition to 0.025 M bicarbonate medium in an atmosphere containing 5 per cent CO_2. For certain experiments the pyocyanine was subjected to further purification as follows: A solution of the hydrochloride was neutralized with $NaHCO_3$, the pyocyanine was extracted with chloroform, and the chloroform solution extracted with 0.1 N HCl. After this transference was repeated several times, the pyocyanine was precipitated from a small volume of dilute acid solution by addition of $NaHCO_3$. It was separated, washed with ice-cold water, dissolved in water at 50°, and allowed to crystallize in the refrigerator. The crystalline base was separated and dried in a vacuum desiccator. A sample of synthetic pyocyanine hydrochloride[1] prepared by the method of Wrede and Strack (1929) was treated similarly.

Effect of Pyocyanine on Oxygen Consumption—Pyocyanine stimulates the respiration of slices of cerebral cortex in oxygen on a substrate of glucose with all concentrations of the dye tested. Cerebral cortex, like kidney tissue, has a low aerobic glycolysis, and Friedheim (1934) showed that, whereas pyocyanine increased the oxygen consumption of kidney tissue in phosphate-Ringer's solution, no increase occurred in bicarbonate-Ringer's solution. In contrast to kidney increased oxidation due to pyocyanine occurs with brain cortex in either a phosphate or a bicarbonate

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[1] The author is indebted to Dr. E. S. West for this material.

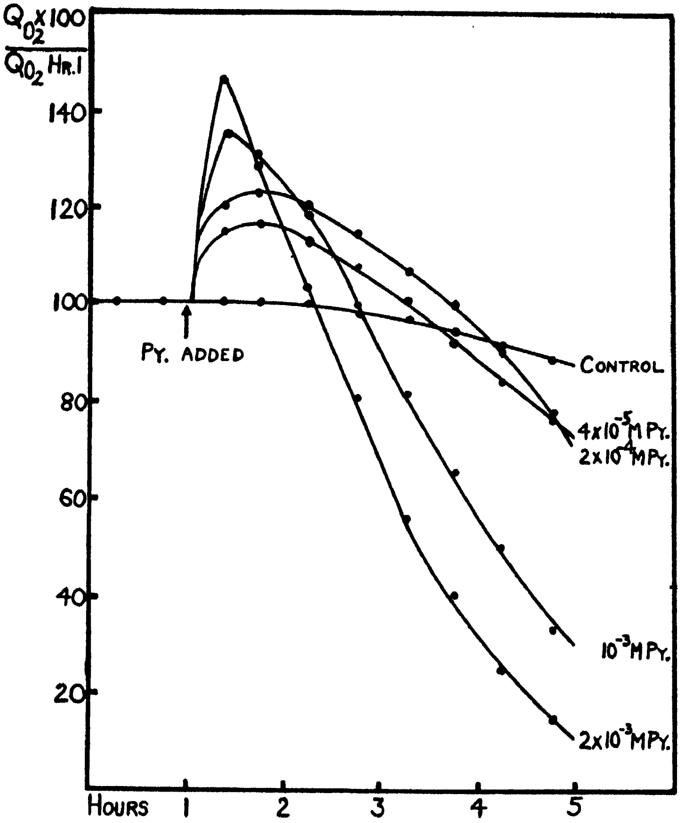

FIG. 1. The effect of different concentrations of pyocyanine on the O_2 consumption rate of rabbit cerebral cortex. Glucose substrate, phosphate medium, atmosphere of O_2.

medium (Table I, Series 1 and 2). The initial rise in the respiration rate of brain cortex increases with increasing concentrations of pyocyanine but in the case of 2×10^{-3} M and 10^{-3} M concentrations this is followed by a rapid inhibition of respiration (Fig. 1). Pyocyanine also increases the respiration of a suspension of brain cortex prepared by grinding the tissue with washed sand (Table I, Series 3). The lower concentrations of pyocyanine are found to be more effective in accelerating oxidation in ground tissue than in slices. In all concentrations of the dye the subsequent inhibition is greater, although the ground tissue controls show a steady oxygen consumption.

Other dyes have been shown to inhibit the respiration of cerebral cortex. For example, Elliott and Baker (1935) found that methylene blue, cresyl violet, and 4,6-dinitro-*o*-cresol in 10^{-5} M concentration increased the respiration of brain cortex on a glucose substrate during a period of 1 hour. In 10^{-3} M concentration, however, these compounds inhibited respiration. The respiration rate of brain cortex in the presence of 10^{-3} M pyocyanine, owing to the lower toxicity of this dye, is still above normal at the end of an hour (Fig. 1). High concentrations of pyocyanine do eventually show a marked inhibitory action on respiration. In order to eliminate the possibility that this is due to a contaminating substance in the pyocyanine, specimens of natural and synthetic pyocyanine hydrochloride were subjected to further purification and the free base was finally obtained in crystalline form from aqueous solution. Solutions of both the purified natural and synthetic products prepared immediately before use show substantially the same inhibitory action and the same as that of the sample of pyocyanine not subjected to this further purification. It seems improbable therefore that this effect is due to impurities in the pyocyanine. The possibility that the extra oxygen consumption induced by pyocyanine is itself responsible for the subsequent inhibition can be excluded on the grounds that a similar inhibition occurs under conditions (*e.g.*, Table I, Series 4) in which there is a slight stimulation of respiration or none.

Quastel and Wheatley (1934) showed that slices of brain tissue can be washed in phosphate-saline-glucose and transferred to a fresh medium with practically no loss in respiration rate, and that when brain slices whose respiration has been strongly inhibited by certain narcotics are so treated, at least 70 per cent of the respiration is recovered. When brain cortex slices which have shown an increased respiration rate due to 10^{-3} M pyocyanine are transferred to a pyocyanine-free medium 2 hours after addition of the dye, no reversibility occurs, and the respiration rate continues to fall. The oxygen consumption of a control tissue is almost unaffected by transference to a fresh medium. Normal

TABLE I

Effect of Pyocyanine on Oxygen Consumption of Cerebral Cortex on a Substrate of Glucose

	Dye concentration	Control period Q_{O_2}, 1 hr.	Dye present $\frac{Q_{O_2} \times 100}{Q_{O_2}, 1\ \text{hr.}}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 1. Phosphate medium, in O_2	2×10^{-3}	11.3	127	91	47	20
	10^{-3}	11.7	126	109	73	40
	2×10^{-4}	12.1	116	116	102	84
	4×10^{-5}	12.2	108	108	98	84
	No dye	11.0	100	99	92	90
Series 2. Bicarbonate medium, in O_2	2×10^{-3}	10.6	122	78	28	8
	10^{-3}	10.2	132	105	60	31
	2×10^{-4}	10.6	112	103	90	81
	4×10^{-5}	9.6	109	110	99	92
	No dye	10.0	86	89	79	78
Series 3. Phosphate medium, in O_2; ground tissue	2×10^{-3}	*	127	60	28	11
	10^{-3}	*	129	67	34	15
	2×10^{-4}	*	135	104	87	47
	4×10^{-5}	*	117	93	72	38
	No dye	9.0	100	102	102	91
Series 4. Phosphate medium, in air	2×10^{-3}	6.5	93	66	45	28
	10^{-3}	5.9	101	85	60	41
	2×10^{-4}	6.7	100	105	94	82
	4×10^{-5}	6.1	96	103	98	92
	No dye	6.4	99	98	94	88
Series 5. Phosphate medium, in air; tissues 2–3 mm. thick	2×10^{-3}	1.9	90	73	61	44
	10^{-3}	2.1	103	92	39	49
	2×10^{-4}	2.4	101	94	75	62
	No dye	2.2	95	90	85	74

* Absolute value not determined. Percentage effect calculated on the basis of the manometric reading for the 1st hour.

brain slices transferred to a pyocyanine medium (10^{-3} M) in which inhibition of respiration of brain cortex tissue has just occurred show the same respiratory stimulation followed by inhibition, showing that no compound which specifically inhibits oxidation is present in the medium as a result of the interaction of the original slices with the pyocyanine.

When air is used in the Warburg respirometers instead of oxygen, the respiration rate of thin slices of brain cortex in phosphate-glucose medium is steady, but about half that obtained when oxygen is used. The rate of diffusion of oxygen is thus a limiting factor for the respiration of cerebral cortex in air. Under these conditions practically no increased oxidation takes place on addition of pyocyanine (Table I, Series 4). The percentage inhibition of respiration which finally occurs in air owing to pyocyanine is about the same as that obtained in oxygen. With thick slices of cerebral cortex (2 to 3 mm.) in air the stimulatory effect of pyocyanine is also slight or non-existent (Table I, Series 5), a finding which is contrary to that obtained with kidney by Friedheim (1934).

Pyocyanine Action with Different Substrates—In the absence of added substrate the respiration of cerebral cortex falls off rapidly, but in the presence of 0.2 per cent glucose, fructose, lactate, or pyruvate (the last two substances being added as sodium salts) the respiration is fairly steady, and about the same value, irrespective of which of these substrates is used (see Table I, Series 1, and Table II). Friedheim (1934) found that substrate (glucose) was necessary for the increase of respiration due to pyocyanine. The findings in the case of cerebral cortex without added substrate are in accordance with this (Table II, Series 6). Comparison of the effects of various substrates on pyocyanine action is complicated by the fact that the substrates do not maintain the respiration rate equally well. Allowing for this fact, some degree of increased oxidation is found initially with all the above substrates, and the comparative stimulatory effect is as follows: glucose > lactate > fructose > pyruvate (Table II). The increased oxygen consumption is better maintained with a glucose substrate than with the others.

Action of Phenazine and Some of Its Derivatives on Oxygen Consumption of Cerebral Cortex—Of the compounds tested only N-methyl-β-oxyphenazine showed a solubility comparable with that of pyocyanine. This compound was therefore tested in the same concentrations as pyocyanine (Table III, Series 10), while the others (phenazine, α- and β-hydroxyphenazine, and α-methoxyphenazine) were used in 2×10^{-4} M concentration (Table III, Series 11). The α form of hydroxyphenazine is considerably more toxic than pyocyanine, whereas the β form shows slightly increased oxidation and practically no inhibitory action. N-methyl-β-oxyphenazine, an isomer of pyocyanine, also gives some increased oxidation and is barely toxic even in high concentrations. Since N-methyl-β-oxyphenazine (E'_0 −0.17 volt at pH 7; Preisler and Hempelmann (1937)) and α-hydroxyphenazine (E'_0 −0.17 volt at pH 7; Michaelis (1931)) have the same oxidation-reduction potential, the inhibition of respiration produced by α-hydroxyphenazine would not appear to be associated with this factor. A low degree of penetration of the dye into the tissue is not responsible for the low inhibitory action of N-methyl-β-oxyphenazine, since it is taken up by the tissues as readily as pyocyanine. The evidence therefore points to an association of the inhibition of respiration with the α-oxy or α-hydroxy group

TABLE II

Effect of Pyocyanine on Oxygen Consumption of Cerebral Cortex without Added Substrate and with Different Substrates (Phosphate Medium, Atmosphere of O_2)

	Dye concentration	Control period Q_{O_2}, 1 hr.	Dye present $\frac{Q_{O_2} \times 100}{Q_{O_2}, 1 \text{ hr.}}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 6. No added substrate	2×10^{-3}	8.8	48	14	6	4
	10^{-3}	8.4	55	20	9	5
	2×10^{-4}	8.5	58	24	11	6
	4×10^{-5}	8.5	62	29	12	7
	No dye	8.6	60	37	21	15
Series 7. Substrate, sodium *d*-lactate	10^{-3}	9.9	112	63	28	13
	2×10^{-4}	10.2	111	97	80	52
	No dye	9.7	98	92	87	86
Series 8. Substrate, sodium pyruvate	10^{-3}	10.1	101	57	19	9
	2×10^{-4}	10.2	96	88	71	46
	No dye	9.6	94	88	86	82
Series 9. Substrate, fructose	10^{-3}	11.1	102	63	31	16
	2×10^{-4}	10.8	105	83	51	31
	No dye	9.9	94	92	83	78

in the phenazine nucleus, since inhibitory action is shown by pyocyanine (N-methyl-4-oxyphenazine) and by α-hydroxyphenazine, but not by N-methyl-β-oxyphenazine and β-hydroxyphenazine. Furthermore, when the α-hydroxy group is masked (as in α-methoxyphenazine) or absent (as in phenazine), the inhibitory action disappears (Table III, Series 11).

With brain cortex N-methyl-β-oxyphenazine causes only a slight increase in oxidation, even though its stimulatory action is not being masked appreciably by a toxic effect. The reason for the quantitative differences in the stimulatory action of pyocyanine (E'_0 −0.035 volt at pH 7; Michaelis, Hill, and Schubert (1932)) and its isomer, N-methyl-β-oxyphenazine (E'_0 −0.17 volt at pH 7; Preisler and Hempelmann (1937)) may possibly be sought in the difference in their oxidation-reduction potentials.

TABLE III

Effect of Various Compounds Related to Pyocyanine on Oxygen Consumption of Cerebral Cortex (Glucose Substrate, Phosphate Medium, Atmosphere of Oxygen)

	Dye concentration	Control period Q_{O_2}, 1 hr.	Dye present $\frac{Q_{O_2} \times 100}{Q_{O_2}\text{, 1 hr.}}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 10. N-Methyl-β-oxyphenazine	2×10^{-3}	12.6	105	102	88	75
	10^{-3}	12.5	102	100	89	78
	2×10^{-4}	12.6	105	107	100	98
	4×10^{-5}	13.2	95	92	87	82
	No dye	11.2	96	92	89	85
Series 11. Phenazine	2×10^{-4}	11.6	95	91	88	86
α-Hydroxyphenazine	2×10^{-4}	11.5	99	86	59	49
β-Hydroxyphenazine	2×10^{-4}	10.0	101	104	100	94
α-Methoxyphenazine	2×10^{-4}	10.7	96	104	98	95
	No dye	11.4	97	92	89	86

Pyocyanine Action in Presence of KCN—Barron (1930) found that methylene blue did not accelerate the respiration of certain mammalian tissues showing no aerobic glycolysis. After the respiration of these tissues had been inhibited by KCN and their fermentative power brought into action, the addition of methylene blue caused increased oxygen consumption. A similar result was obtained by DeMeio, Kissin, and Barron (1934) using carbon monoxide for the inhibition of respiration. Friedheim (1931) observed that the action of pyocyanine in increasing the respiration of *Bacillus pyocyaneus* was dependent on the presence of one or more respiratory ferments sensitive to KCN and CO. He further showed that the reduction of pyocyanine and reoxidation of leucopyocyanine were not impeded by KCN or CO.

Table IV

Effect of Pyocyanine on Oxygen Consumption of Cerebral Cortex in Presence of 0.001 m KCN or 0.1 m KCl (Glucose Substrate, Phosphate Medium, Atmosphere of O_2)

	Dye concentration	Control period Q_{O_2}, 1 hr.	Dye present $\frac{Q_{O_2} \times 100}{Q_{O_2}, 1 \text{ hr.}}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 12. 0.001 M KCN present throughout	2×10^{-3}	2.6	74	27	19	
	10^{-3}	2.1	85	35	28	
	2×10^{-4}	2.6	85	32	20	
	4×10^{-5}	2.2	97	34	19	
	No dye	2.2	53	28	22	
Series 13. 0.1 M KCl added with the dye	2×10^{-3}	9.0	116	72	33	17
	10^{-3}	9.3	130	86	46	22
	2×10^{-4}	9.6	150	117	74	40
	4×10^{-5}	9.8	144	128	94	62
	No dye	9.5	130	118	97	85

With 0.001 M and 0.01 M KCN the initial inhibition of brain cortex respiration is about 80 and 90 per cent respectively[2] (*cf.* Dixon and Elliott (1929), van Heyningen (1935)). The inhibition increases with time, and in the presence of 0.001 M KCN the addition of pyocyanine merely retards this process somewhat (Table IV, Series 12). On a percentage basis the respiration of cyanide-treated cerebral cortex, during the 1st hour after addition

of pyocyanine, is much higher than that of cyanide-treated tissue to which dye is not added. The difference in actual oxygen consumption is small, however, and except in the case of 4×10^{-5} M pyocyanine, it is much less than that obtained in the absence of cyanide. While these results indicate a dependence of the pyocyanine action on cyanide-sensitive oxidation systems, it is probable that some factor is inhibiting the action of the dye under these conditions, since an increasing effect on respiration is obtained with decreasing concentrations of pyocyanine.

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[2] KCN solutions were neutralized with HCl before use. Reduction in the concentration of cyanide in the medium due to its absorption by the alkali in the inset was avoided by using the appropriate KCN-KOH mixtures (Krebs, 1935) for the absorption of CO_2.

Pyocyanine Action in Presence of 0.1 M KCl—Ashford and Dixon (1935) found that 0.1 M KCl increased the respiration and aerobic glycolysis of brain tissue, an effect which has been ascribed to the action of KCl on permeability and dispersion of cell colloids (Dickens and Greville, 1935; Dixon and Holmes, 1935; Dixon, 1936). It appeared of interest to know whether or not the increased respiration due to pyocyanine is superimposable on that due to high concentrations of KCl. When 2×10^{-3} M pyocyanine and 0.1 M KCl are added together, the resulting increase in respiration is less than that produced by the addition of either of them separately (Table IV, Series 13). With either 2×10^{-4} M or 4×10^{-5} M pyocyanine added with 0.1 M KCl, however, the increased respiration rate is greater than that due to either the salt or the dye alone, and is approximately additive at the time of maximum stimulation (Fig. 2, *A* and *B*).

Pyocyanine and Aerobic Glycolysis—Friedheim (1934) showed that pyocyanine (2×10^{-3} M) decreased the aerobic glycolysis as well as increased the respiration of a number of tumors. Cerebral cortex has a low rate of aerobic glycolysis and even this tends to decrease with time. Measurements of the aerobic acid production of brain cortex show that this is little affected by low concentrations of pyocyanine (2×10^{-4} M and 4×10^{-5} M). With 2×10^{-3} M and 10^{-3} M pyocyanine a progressive rise in aerobic acid production results (Table V, Series 14), and lactic acid determinations show that the acid produced is in all probability lactic acid (Table V). The increased glycolysis is not initially due to inhibition of respiration, since the onset occurs during the period of increased oxidation. In this respect the action of pyocyanine is like that found with brilliant cresyl blue (E'_0 +0.045 volt at pH 7; Cohen and Preisler (1931)) on brain cortex by Dickens (1936).

Pyocyanine and Anaerobic Glycolysis—With tumor tissue Dickens (1936) found that in the presence of nitrogen containing traces of oxygen pyocyanine brought about an inhibition of anaerobic glycolysis, provided the concentration of pyocyanine was sufficient for some of it to remain oxidized throughout the experiment. When traces of oxygen were absent and complete decoloration of the pyocyanine occurred, there was little or no inhibition of anaerobic glycolysis in tumor tissue. With brain cortex it

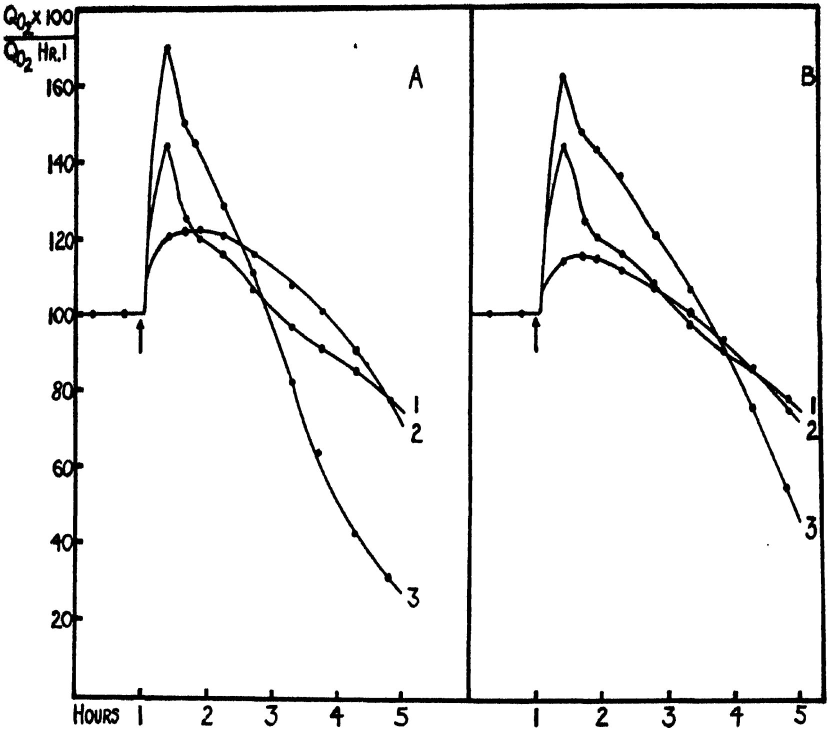

FIG. 2. The effect of adding pyocyanine or 0.1 M KCl or both on the O_2 consumption rate of rabbit cerebral cortex. *A*, Curve 1, 0.1 M KCl; Curve 2, 2×10^{-4} M pyocyanine; Curve 3, 0.1 M KCl and 2×10^{-4} M pyocyanine. *B*, Curve 1, 0.1 M KCl; Curve 2, 4×10^{-5} M pyocyanine; Curve 3, 0.1 M KCl and 4×10^{-5} M pyocyanine. The additions were made at the points marked by the arrows. Glucose substrate, phosphate medium, atmosphere of O_2.

has been found that irrespective of the presence or absence of traces of oxygen pyocyanine increases the anaerobic glycolysis initially at all concentrations of the dye tested (Table VI). With 2×10^{-3} M pyocyanine with about 10 mg. (dry weight) of tissue under strictly anaerobic conditions oxidized dye is still present after 4 hours. With 10^{-3} M, 2×10^{-4} M, and 4×10^{-5} M pyocyanine decoloration occurs within approximately 90, 30, and 15 minutes respectively. In the presence of traces of oxygen the medium remains blue throughout the 4 hours of the experiment with all the above concentrations of pyocyanine. Under

TABLE V

Effect of Pyocyanine on Aerobic Acid Production by Cerebral Cortex on Substrate of Glucose

	Dye concentration	Control period $Q_A^{O_2}$, 1 hr.	Dye present $Q_A^{O_2}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 14. Bicarbonate medium; in O_2 containing 5% CO_2	2×10^{-3}	+1.5	+2.7	+3.2	+5.0	+5.5
	10^{-3}	+1.8	+2.1	+2.4	+3.7	+4.6
	2×10^{-4}	+1.5	+0.8	+0.5	+0.6	+0.7
	4×10^{-5}	+2.9	+1.3	+1.2	+0.9	+1.7
	No dye	+2.1	+1.1	+0.2	+0.1	+0.3

The term $Q_A^{O_2}$ represents the aerobic acid production in terms of c.mm. of CO_2 liberated from the bicarbonate medium per hour, per mg. of dry weight of tissue.

Chemical Determination of Lactic Acid (Glucose Substrate, Phosphate Medium, Atmosphere of O_2)

	Lactic acid per gm. dry weight of tissue	
	Rabbit A	Rabbit B
	mg.	*mg.*
At time of dye addition	23	25
After 2 hrs. 2×10^{-3} M pyocyanine .	47	
" 2 " 10^{-3} M " .	40	40
" 2 " 2×10^{-4} M " .	33	30
" 2 " no pyocyanine.	28	29

strictly anaerobic conditions and in the presence of traces of oxygen, the two highest concentrations of the dye cause an inhibition of glycolysis following the initial increase. With the two lowest concentrations of pyocyanine an increased rate of glycolysis is maintained, irrespective of the presence or absence of traces of oxygen. Decoloration of the dye in these low concentrations is so rapid under strictly anaerobic conditions that the effects observed for the greater part of the experiment are due to the reduced form of pyocyanine. Since oxidized dye is present in the medium throughout the experiment when traces of oxygen are present, it would appear that in low concentrations both oxidized and reduced pyocyanine increase the anaerobic glycolysis of cerebral cortex.

TABLE VI

Effect of Pyocyanine on Anaerobic Glycolysis of Cerebral Cortex in Presence and Absence of Traces of Oxygen (Glucose Substrate, Bicarbonate Medium, Atmosphere of 5 Per Cent CO_2 in N_2)

	Dye concentration	Control period $Q_G^{N_2}$, 1 hr.	Dye present $\frac{Q_G^{N_2} \times 100}{Q_G^{N_2}\text{, 1 hr.}}$			
			2nd hr.	3rd hr.	4th hr.	5th hr.
	M					
Series 15. Trace of O_2 present	2×10^{-3}	12.3	67	15	5	3
	10^{-3}	10.5	80	41	19	9
	2×10^{-4}	11.3	86	70	57	45
	4×10^{-5}	10.6	80	65	48	37
	No dye	10.4	63	49	36	30
Series 16. No oxygen present	2×10^{-3}	9.4	80	26	11	6
	10^{-3}	11.1	89	38	15	8
	2×10^{-4}	10.1	83	64	47	37
	4×10^{-5}	9.5	76	59	44	35
	No dye	10.5	62	43	32	26

DISCUSSION

According to Barron (1929) the catalytic action of oxidation-reduction dyes on cellular oxidations is due to the oxidation of easily oxidizable carbohydrates, the reduced dye so formed being reoxidized by atmospheric oxygen. The catalysis is influenced by various properties of the dye, its oxidation-reduction potential and degree of penetration into the cell (Barron and

Hoffman, 1930), and its toxicity (Quastel and Wheatley, 1931). Barron (1930) did not find that methylene blue increased oxidation in tissues which did not show aerobic glycolysis. Since a dye-catalyzed respiration was observed in such tissues after their normal respiration had been inhibited by KCN (Barron, 1930) or CO (DeMeio, Kissin, and Barron, 1934), it was concluded by these workers that "reversible dyes act as catalysts for the oxidation of the lactic acid produced by the tissues."

Pyocyanine can accelerate glycolytic processes in cerebral cortex. This is seen in its action on anaerobic glycolysis, and in the increased aerobic glycolysis which high concentrations of the dye produce while stimulating respiration. In view of this effect on glycolytic mechanisms the possibility arises that the increased respiration of cerebral cortex due to pyocyanine may be mainly a result of the dye accelerating glycolysis and catalyzing oxidation of the lactic acid so produced. It is not known, however, the extent to which other factors (*e.g.*, the rate of activation of lactic acid) might limit such an action of the dye. Furthermore, there is evidence which indicates that glucose may not necessarily be converted to lactic acid before its oxidation can be catalyzed by pyocyanine. Pyocyanine brings about a greater increase of respiration on a glucose substrate than on lactate, although in the absence of the dye the respiration rate on a lactate substrate is about the same or slightly lower than on a substrate of glucose. Also it has been shown that pyocyanine can catalyze oxidations on substrates other than glucose or lactate. Thus some increased oxidation is observed on a substrate of fructose, from which little or no lactic acid is produced in brain (Loebel, 1925; Dickens and Greville, 1932). It is possible, therefore, that pyocyanine exerts its oxidative action on some degradation product of glucose other than lactic acid. In this connection it is of interest to note that Jowett and Quastel (1937) have shown that glucose can be oxidized in brain by a mechanism which does not involve lactate as an intermediary.

SUMMARY

1. A study has been made of the effect of pyocyanine in 2×10^{-3} M, 10^{-3} M, 2×10^{-4} M, and 4×10^{-5} M concentrations on the metabolism of slices of rabbit cerebral cortex.

2. All concentrations of the dye tested accelerate respiration on a glucose substrate in an atmosphere of oxygen. The increased oxygen consumption is followed by an irreversible inhibition of respiration.
3. Pyocyanine also initially increases the respiration of cerebral cortex on substrates of lactate, fructose, and pyruvate, but to a less extent than on a substrate of glucose.
4. Pyocyanine does not accelerate respiration in the absence of added substrate, and only a slight increase or none is observed with a glucose substrate in air.
5. The action of phenazine and some of its derivatives related to pyocyanine indicates that inhibition of respiration is associated with the presence of an α-oxy or α-hydroxy group in the phenazine nucleus.
6. Concentrations of pyocyanine above 4×10^{-5} M produce less increase in oxygen consumption in the presence of 0.001 M KCN than in its absence.
7. In the presence of 0.1 M KCl the increases in respiration due to 2×10^{-4} M and 4×10^{-5} M pyocyanine are approximately the same as those which they produce in the absence of 0.1 M KCl.
8. Pyocyanine, in 2×10^{-3} M and 10^{-3} M concentrations, increases the aerobic glycolysis of cerebral cortex while causing increased oxygen consumption. The dye in 2×10^{-4} and 4×10^{-5} M concentrations has little effect on aerobic glycolysis.
9. All concentrations of pyocyanine tested increase anaerobic glycolysis initially, but the effect is maintained only by the two lower concentrations of the dye.
10. These findings are discussed in the light of the views advanced by Barron and his coworkers for the mechanism of the catalytic action of oxidation-reduction dyes on tissue oxidations.

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BIBLIOGRAPHY

Ashford, C. A., and Dixon, K. C., *Biochem. J.*, **29**, 157 (1935).
Barron, E. S. G., *J. Biol. Chem.*, **81**, 445 (1929); *J. Exp. Med.*, **52**, 447 (1930).
Barron, E. S. G., and Harrop, G. A., Jr., *J. Biol. Chem.*, **79**, 65 (1928).
Barron, E. S. G., and Hoffman, L. A., *J. Gen. Physiol.*, **13**, 483 (1930).

Cohen, B., and Preisler, P. W., *Pub. Health Rep., U. S. P. H. S.*, suppl. 92 (1931).

DeMeio, R. H., Kissin, M., and Barron, E. S. G., *J. Biol. Chem.*, **107**, 579 (1934).

Dickens, F., *Biochem. J.*, **30**, 1064 (1936).

Dickens, F., and Greville, G. D., *Biochem. J.*, **26**, 1546 (1932); **29**, 1468 (1935).

Dixon, K. C., *Nature*, **137**, 742 (1936).

Dixon, K. C., and Holmes, E. G., *Nature*, **135**, 995 (1935).

Dixon, M., Manometric methods, Cambridge (1934).

Dixon, M., and Elliott, K. A. C., *Biochem. J.*, **23**, 812 (1929).

Elema, B., and Sanders, A. C., *Rec. trav. chim. Pays-Bas*, **50**, 796 (1931).

Elliott, K. A. C., and Baker, Z., *Biochem. J.*, **29**, 2396 (1935).

Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, **73**, 335 (1927).

Friedheim, E. A. H., *J. Exp. Med.*, **54**, 207 (1931); *Biochem. J.*, **28**, 173 (1934).

Harrop, G. A., Jr., and Barron, E. S. G., *J. Exp. Med.*, **48**, 207 (1928).

van Heyningen, W. E., *Biochem. J.*, **29**, 2036 (1935).

Jowett, M., and Quastel, J. H., *Biochem. J.*, **31**, 275 (1937).

Krebs, H. A., *Biochem. J.*, **29**, 1620 (1935).

Loebel, R. O., *Biochem. Z.*, **161**, 219 (1925).

Michaelis, L., *J. Biol. Chem.*, **92**, 211 (1931).

Michaelis, L., Hill, E. S., and Schubert, M. P., *Biochem. Z.*, **255**, 66 (1932).

Preisler, P. W., and Hempelmann, L. H., *J. Am. Chem. Soc.*, **59**, 141 (1937).

Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **25**, 629 (1931); **28**, 1521 (1934).

Warburg, O., *Biochem. Z.*, **142**, 317 (1923); Über den Stoffwechsel der Tumoren, Berlin (1926).

Wendel, W. B., *J. Biol. Chem.*, **102**, 47 (1933).

Wrede, F., and Strack, E., *Z. physiol. Chem.*, **181**, 58 (1929).

CHEMICAL CONSTITUTION OF ENAMEL AND DENTIN

I. PRINCIPAL COMPONENTS*

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An understanding of the calcification process requires exact knowledge of the nature and variations of composition of the mineral phase of calcified tissues. Other investigators have continued the study, begun years ago, of the composition of the inorganic salts of bone, but a comprehensive study of the results of analysis of enamel and dentin by modern methods has not been described. Crowell, Hodge, and Line (1934) have reported the results of a very complete survey of the composition of the mineral phase of whole adult teeth. These authors present a comprehensive summary of the literature on the composition of whole teeth, enamel, and dentin. Recently Le Fevre, Bale, and Hodge (1937) described the composition of the mineral phase of whole fetal teeth. The analyses reported in both papers were made on the inorganic residue prepared by boiling the specimen in a solution of potassium hydroxide and ethylene glycol.

The variations in relative amounts of enamel, dentin, and cementum in teeth make the interpretation of analyses of whole teeth difficult and it is, therefore, desirable to determine the composition of enamel and dentin separately. The older studies described the composition of only a few specimens and in many cases the analyses were made on ashed materials. Changes in composition of the mineral phase are known to result during ignition (Bowes and Murray, 1935; Howland, Marriott, and Kramer, 1931). In recent years only a few attempts have been made to analyze enamel and dentin. Bowes and Murray (1935, 1936) have reported the composition of the pooled enamel and dentin of a number of human teeth. Logan (1935) presented the analyses of the enamel and dentin fractions of two human and two dog teeth and compared the results with those of bone obtained by the same methods. The paucity of information relative to the composition of these fractions of teeth is undoubtedly due to the difficulty experienced in their quantitative separation. Other workers separated enamel and dentin by grinding or other laborious and uncertain mechanical procedures. The method previously described (Brekhus and Armstrong, 1935) by us has permitted a more ready separation of enamel and dentin from each other and from cementum.

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* An abstract of this work was reported at the meeting of the American Society of Biological Chemists at Detroit, April 10, 1935 (Armstrong, 1935). It was supported by a grant from the Graduate Medical Research Fund of the University of Minnesota.

We have completed the analyses of a sufficient number of specimens to describe, with statistical significance, the normal variations of composition of enamel and dentin. The possible difference in the composition of enamel with respect to the principal elements of sound and carious teeth has been investigated. The relationship of composition of enamel to the varying susceptibility of the teeth of the human dentition to decay has been studied. Other factors which might be expected to influence the amounts of the constituents of enamel have been considered. The analytical procedures were applied to the unashed protein-containing specimens to eliminate the possibility of chemical alteration of the material in preparation for analysis. Such analyses can be directly compared with those of bone made by Kramer and Shear (1928).

Methods

The teeth, immediately following extraction, were stored in 95 per cent alcohol until preliminary procedures were applied. The lesions of carious teeth were entirely ground away until only sound enamel and dentin remained, since to have left the decayed areas in place would have resulted in the determination of alterations of composition secondary to the carious process. The specimens were cleaned with the aid of dental instruments, broken into bits, and the pulp removed. The fragments were made into a packet in cloth and extracted for 6 hours, in a Sohxlet apparatus, with a mixture of equal parts of alcohol and ether. Each specimen was dried at 60°, pulverized until all passed a 100 mesh sieve, and the powder submitted to the enamel and dentin separation process (Brekhus and Armstrong, 1935).

With certain exceptions, the analytical procedures[1] are modifications of those used by Kramer and Howland (1926) and Shear and Kramer (1928) for the analysis of bone. Approximately 50 mg. of enamel or 70 mg. of dentin were put in solution in 20 cc. of M HCl by heating the flask in a boiling water bath for 15 minutes. The solution was diluted to 100 cc., that of dentin being first filtered through retentive paper to remove a slight cloudiness, probably caused by undissolved protein.

Calcium was determined in duplicate on 2 cc. aliquots of the solution according to the method of Kramer and Tisdall. The supernatant liquid was siphoned from the precipitate of calcium oxalate which was then washed twice with dilute ammonium hydroxide solution. Potassium permanganate (0.01 N), standardized against sodium oxalate from the Bureau of Standards, was employed. It is important to determine separate blanks for the titration of the standard oxalate solutions and unknowns if the final volumes of the titrated solutions of the standards are considerably greater than those of the unknowns, since the amount of excess permanganate required to color the solutions varies directly as their volumes. Ten analyses of solutions containing 0.446 mg. of calcium per 2 cc. were made. The largest error was + 2 per cent and the error of six results was +0.7 per cent of the calculated amount. Five samples of enamel were analyzed in the routine manner and several months later five determinations were made on each sample. The greatest difference between the first and the average of the three most concordant of the subsequent determinations on any one specimen was 1.4 per cent.

Phosphorus was determined in duplicate on 5 cc. aliquots of the solution by the gravimetric method of Embden (1921) which employs the precipitation of strychnine phosphomolybdate. It is important to use not more than 3 drops of brom-phenol blue solution, added after the aliquots are diluted to 30 cc., since an excess of indicator causes high results. The troublesome formation of cracks in the precipitate in the Gooch crucible, which hinders adequate washing, is prevented if the suction is regulated at 100 mm. of mercury and the precipitate kept wet with wash liquid. Precipitates containing less than 0.5 mg. of phosphorus rarely develop cracks with any form of treatment. Solutions containing 0.5 mg. of phosphorus were analyzed with errors not greater than 1 per cent.

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[1] A detailed description of the analytical methods used in this study has been published (McClendon and Pettibone, 1936).

Magnesium was determined in duplicate on 20 cc. aliquots of the solution by an adaptation of the Briggs (1922) method. In the case of enamel and dentin, a quantitative separation of calcium and magnesium oxalates is not obtained at pH 6.2, as described by Kramer and Howland (1926) for bone. In solutions containing a large excess of calcium, the precipitation of calcium oxalate at about pH 4 (just pink to methyl red) permits the most accurate results to be obtained on the analysis for known amounts of magnesium.

To each aliquot 2 drops of methyl red solution and 1 cc. of 0.5 M oxalic acid were added. The solution was heated to boiling and M NH_4OH added until the color was yellow-pink. Very dilute HCl was then added until the color was light pink, following which the mixture was digested on a steam bath for 3 hours. The precipitate of calcium oxalate was removed by filtration through Whatman No. 42 paper, the beaker and filter being washed with four 10 cc. volumes of 0.1 M NH_4OH. The filtrate was evaporated to about 7.5 cc., transferred to a pointed 50 cc. centrifuge tube, and the determination finished in the manner described by Kramer and Howland. The magnesium ammonium phosphate precipitate, which was allowed to collect overnight in a refrigerator, was washed with four 10 cc. volumes of 3 M NH_4OH. The supernatant fluid was removed, after centrifugation, with the aid of a siphon. The standard solutions contained 0.05 and 0.2 mg. of phosphorus for the analysis of enamel and dentin respectively. All solutions were diluted to 50 cc. for colorimetric analysis.

Numerous determinations of the magnesium content of solutions containing 0.15 mg. of magnesium, 10 mg. of phosphorus, 15 mg. of calcium, and 20 cc. of M HCl per 100 cc. gave results which were within 5 per cent of the theoretical content.

Carbonate was determined as carbon dioxide on separate 40 to 50 mg. samples in the Van Slyke volumetric apparatus. The sample was washed into the chamber of the apparatus with 4 cc.

of water divided into small portions, and another 1 cc. was added to overlay the material. The fluid level in the gas burette was raised until it entered the bore of the stop-cock of the filling bulb and the stop-cock was closed. Then 1 cc. of water was placed in the filling bulb and underlaid with 5 cc. of 5 N HCl which was rapidly admitted to contact with the sample by opening the upper stop-cock, after producing a slight vacuum in the chamber. The determination was finished in the manner described by Kramer and Howland (1926). Since 10 cc. of liquid were present in the chamber of the apparatus, the calculation of the result according to the formulas of Van Slyke (1917) (*cf.* Kramer and Howland, 1926) was simplified. Attempts were made to use the solution removed from the Van Slyke apparatus for the determination of calcium, magnesium, and phosphorus but the results did not agree with those made directly on the sample. The quantity of enamel obtainable from a tooth is limited and not all determinations of carbonate on this material were done in duplicate.

Nitrogen was determined in duplicate on separate samples of dentin by the Kjeldahl method according to the technique of Cavett (1931). The determinations of nitrogen in enamel, which required nesslerization of ammonia, are not reported because the sample limitations permitted only a few determinations, whose results were variable.

The mean differences in duplicate analyses of enamel when the results are expressed as percentage of the sample were as follows: calcium 0.189, phosphorus 0.087, magnesium 0.025, and carbon dioxide 0.044. These differences in the analyses of dentin were calcium 0.100, phosphorus 0.039, magnesium 0.022, carbon dioxide 0.034, and nitrogen 0.058. Duplicate analyses which failed to check within the usual range were repeated wherever possible.

Results

Table I is a summary[2] of all results of analysis of sound enamel and dentin. Most of the analyses were made on the enamel and dentin fractions of a single tooth, but in some instances it was necessary to pool paired teeth from one individual to increase the amount of enamel to a quantity which would permit all analytical procedures to be applied.

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[2] The data of the analyses of the individual specimens, identified as to sex and age of the person and position of the tooth in the mouth, will be supplied to those wishing them. The composition of the enamel and dentin fractions of the same teeth can then be compared.

Composition of Mineral Phase of Enamel and Dentin—In Table II are presented certain conventional calculations based on the data of Table I. Practically every publication on the chemical composition of calcified tissues includes such calculations, but it should be realized that the concept of state of combination of the elements implied by these ratios is fictitious. It will be noted that the residual Ca to total P ratio of enamel and dentin does not equal that of the hypothetical $Ca_3(PO_4)_2$, namely 1.94, as is the case with bone (Shear and Kramer, 1928). The high magnesium content of these materials requires that the phosphorus calculated to be in combination as $Mg_3(PO_4)_2$ be considered if the above ratio is to approach 1.94.

TABLE I

Analyses of Enamel and Dentin of Sound Teeth

	Enamel			Dentin		
	Mean	Standard deviation	No. of analyses	Mean	Standard deviation	No. of analyses
	per cent	*per cent*		*per cent*	*per cent*	
Ca	35.41	0.963	42	26.18	0.342	20
P	17.45	0.513	42	12.74	0.482	20
Mg	0.30	0.054	34	0.83	0.083	20
CO_2	3.00	0.249	41	3.57	0.103	20
N				3.36	0.145	20

With the exception of magnesium, the constituents of dentin vary less than those of enamel, as denoted by the lower values of the standard deviation of the means for dentin. This observation may find an explanation in the fact that dentin retains, after the eruption of the tooth, a mechanism, far superior to that of enamel, for interchange between itself and the circulating fluids. Opportunity is thereby afforded throughout the life of the dental pulp for adjustment of the composition of dentin towards a uniform content of each element.

The magnesium and carbonate contents of dentin are greater than those of enamel in spite of the very much larger protein and hence smaller mineral content of dentin. The mean protein content of dentin is 22.24 per cent (Armstrong, Brekhus, and Cavett, 1936), while that of enamel is certainly less than 1 per cent. The calcium and phosphorus contents of dentin, while lower than those of enamel, do not stand in direct relationship to the difference in the protein contents of the two materials, as would be the case were the inorganic phases of enamel and dentin identical. For these reasons, it was considered (Armstrong, 1935; Brekhus and Armstrong, 1935, *b*) that the elementary composition of the mineral phase of enamel differs from that of dentin. In order to demonstrate more clearly the differences in the analytical composition of the inorganic phases of enamel and dentin, which are partially obscured when the analyses are made on the protein-containing material, the experiment whose results are shown in Table III was performed. The pooled enamel and dentin fractions of four teeth were analyzed directly. The protein-free inorganic residue of each lot of material was prepared according to the modification developed by Crowell, Hodge, and Line (1934) of Gabriel's original method. The analytical composition of the protein-free mineral phase of enamel is obviously different from that of dentin.

TABLE II

Ratios of Elements of Mineral Phases of Enamel and Dentin

	Enamel	Dentin
$\frac{\%\text{Ca}}{\%\text{P}}$	2.03	2.05
$\frac{\text{Residual Ca*}}{\text{Total P}}$	1.87	1.80
$\frac{\text{Residual Ca}}{\text{Residual P†}}$	1.90	1.90
$\frac{\%Ca_3(PO_4)_2}{\%CaCO_3}$	12.60	7.41

* Residual Ca = per cent total Ca minus per cent CO_2 × 0.91 = Ca uncombined as $CaCO_3$.

† Residual P = per cent total P minus per cent Mg × 0.86 = P uncombined as $Mg_3(PO_4)_2$.

The physicochemical conditions required for calcification can be studied better if the nature of the products of this process are known, and it is customary to derive, from the results of analyses, empirical formulas which purport to describe the character of the inorganic salt of calcified tissues. Morgulis (1931), among others, has derived such a formula for bone, and Crowell, Hodge, and Line (1934) tentatively proposed the formula $Ca(OH)_2 \cdot CaCO_3 \cdot 3Ca_3(PO_4)_2$ for the inorganic residue of mixtures of enamel, dentin, and cementum. The *empirical* formulas $MCO_3 \cdot [M_3(PO_4)_2]_{4.1}$ and $MCO_3[M_3(PO_4)_2]_{2.5}$ for enamel and dentin, respectively, almost exactly fit the means of the analytical data. In these formulas M represents calcium and magnesium, the atomic proportions of these elements being 76:1 and 19.1:1 in enamel and dentin, respectively.

TABLE III

Comparison of Composition of Enamel and Dentin and Their Mineral Phases

	Dentin	Enamel	Mineral phase of dentin	Mineral phase of enamel
	per cent	*per cent*	*per cent*	*per cent*
Ca	25.86	36.10	33.69	36.41
P	12.53	17.31	15.78	17.39
Mg	0.73	0.23	0.89	0.24
CO_2	3.64	3.05	4.76	3.07
N	3.43	0.098	0.00	0.00

These formulas, in spite of their agreement with the analytical results, cannot represent the true state of combination of the elements in enamel and dentin. If the formula of the mineral phase of any calcified tissue is to be correctly represented, it is necessary to account for the fact that the x-ray spectrograms of the mineral phases of all calcifications are practically identical with each other and with pure apatites (Taylor and Sheard, 1929). On the other hand, in view of the analytical differences between enamel and dentin and the other calcifications, the data of x-ray analysis cannot be interpreted to indicate an exactly identical composition of the mineral phases of these materials.

An explanation is needed of the fact that several substances may exhibit almost identical x-ray diffraction patterns and yet their compositions be so diverse as to appear irreconcilable by the substitution of equivalent atoms in the apatite crystal lattice, as it is now conceived. Until such explanation is forthcoming, attempts to depict the composition of the inorganic fraction of bone, enamel, and dentin as a single molecular species are futile. These results emphasize that the composition of the mineral deposited in calcified tissues is not absolutely fixed.

Relation of Composition of Enamel to Decay—Table IV is a summary of the analyses of fifteen specimens of enamel of carious teeth. The means of the results and the standard deviations of the means are not significantly different from those of enamel of sound teeth. No significant difference could be detected in the composition of the enamel of sound and carious teeth of the same person. The age of the persons from whom the carious teeth were obtained varied from 20 to 49 years. Specimens of each type of tooth were studied and in some instances it was necessary to pool the enamel of homologous teeth from one person to obtain sufficient material for analysis. These results indicate that no significant alteration is produced in enamel, with respect to the content of the principal elements, beyond the site of the carious lesion.

TABLE IV

Composition of Enamel of Carious Teeth

	Mean	Standard deviation	No. of analyses
	per cent	*per cent*	
Ca	35.64	0.598	15
P	17.21	0.398	15
Mg	0.32	0.053	15
CO_2	3.01	0.145	14

Brekhus (1931) has determined the incidence of caries in the several teeth of the human dentition, and it is possible that there are differences in composition of the enamel of sound teeth which could be correlated with the known individual susceptibility of the tooth types of decay. A rigid test of this hypothesis would require the analysis of the enamel fractions of a number of sound, newly erupted permanent teeth. It has been possible to obtain only a few specimens of sound teeth from youths. The data of Table I were rearranged in three groups according to the resistance of the tooth types to decay. The mean calcium contents of the groups, in order of susceptibility to decay, were 35.14, 35.85, and 35.07 per cent. These means were compared for significant differences by "Student's" method. The calculated probability of the high calcium content of the enamel of the moderately susceptible group being a result of random sampling is less than 3 chances in 100. Except for calcium, there was no possibility of real differences in composition of the three groups. Because of the small and irregular differences in calcium content, and because of the few specimens in each group, it is not possible to conclude that the caries resistance of the tooth types constituting each group is related to the calcium content of the enamel.

A summary of the results of separate analysis of fourteen specimens of enamel prepared from the teeth of patient A is shown in Table V. Since the enamel fractions of homologous teeth were pooled in many instances, the composition of the enamel of twenty teeth is represented in the means. As indicated by the values of the standard deviation of the means, the composition of the enamel of this person varied about as much as that of all other specimens considered together. Patient A was 48 years old and his teeth were unusually free of caries. There were no certain differences of composition of the enamel of these teeth in comparison with that of the sound teeth of other persons or with that of carious teeth which could account for their resistance to decay.

TABLE V

Composition of Enamel of Teeth of One Person Compared with That of Several Persons

	Enamel of teeth of patient A			Enamel of all other teeth		
	Mean	Standard deviation	No. of analyses	Mean	Standard deviation	No. of analyses
	per cent	*per cent*		*per cent*	*per cent*	
Ca	35.86	0.540	14	35.19	0.903	28
P	17.60	0.566	14	17.38	0.210	28
Mg	0.28	0.028	13	0.31	0.073	21
CO_2	3.01	0.230	14	3.00	0.190	27

No definite relationship could be discovered between the composition of enamel and the age of eruption of the teeth.

SUMMARY

1. Variations of composition of enamel and dentin have been described.

2. Since the magnesium and carbonate contents of dentin are higher than those of enamel, and because of other differences in composition, it is concluded that the mineral phases of enamel and dentin are not identical.

3. The composition of the enamel of carious teeth does not differ with respect to the elements determined from that of sound teeth.

4. There has been discovered no correlation of composition of enamel with susceptibility to decay or with the age of eruption of the teeth.

5. The composition of the enamel of the teeth of one person varies as much as that obtained from the teeth of several individuals.

BIBLIOGRAPHY

Armstrong, W. D., *Proc. Am. Soc. Biol. Chem.*, **8**, iv (1935) (*J. Biol. Chem.*, **109** (1935)).

Armstrong, W. D., Brekhus, P. J., and Cavett, J. W., *J. Dent. Research*, **15**, 312 (1936).

Bowes, J. H., and Murray, M. M., *Biochem. J.*, **29**, 2721 (1935); *Brit. Dent. J.*, **61**, 473 (1936).

Brekhus, P. J., *J. Am. Dent. Assn.*, **18**, 1350 (1931).

Brekhus, P. J., and Armstrong, W. D., *J. Dent. Research*, **15**, 23 (1935, *a*); **15**, 164 (1935, *b*).

Briggs, A. P., *J. Biol. Chem.*, **52**, 349 (1922).

Cavett, J. W., *J. Lab. and Clin. Med.*, **17**, 79 (1931).

Crowell, C. D., Hodge, H. C., and Line, W. R., *J. Dent. Research*, **14**, 251 (1934).

Embden, G., *Z. physiol. Chem.*, **113**, 138 (1921).

Howland, J., Marriott, W. McK., and Kramer, B., *J. Biol. Chem.*, **68**, 721 (1931).

Kramer, B., and Howland, J., *J. Biol. Chem.*, **68**, 711 (1926).

Kramer, B., and Shear, M. J., *J. Biol. Chem.*, **79**, 147 (1928).

Le Fevre, M. L., Bale, W. F., and Hodge, H. C., *J. Dent. Research*, **16**, 85 (1937).

Logan, M. A., *J. Biol. Chem.*, **110**, 375 (1935).

McClendon, J. F., and Pettibone, C. J. V., Physiological chemistry, St. Louis, 6th edition, 335–339 (1936).

Morgulis, S., *J. Biol. Chem.*, **93**, 455 (1931).

Shear, M. J., and Kramer, B., *J. Biol. Chem.*, **79**, 105 (1928).

Taylor, N. W., and Sheard, C., *J. Biol. Chem.*, **81**, 479 (1929).

Van Slyke, D. D., *J. Biol. Chem.*, **30**, 347 (1917).

THE SPEED WITH WHICH VARIOUS PARTS OF THE BODY REACH EQUILIBRIUM IN THE STORAGE OF ETHYL ALCOHOL

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That administered alcohol penetrates into every tissue and fluid of the body has been repeatedly demonstrated following the pioneer work of Grehant, which began in 1895 (1). The distribution of this alcohol, after equilibrium occurs, has been studied by Grehant (1), Nicloux (2), Mellanby (3), Carpenter (4), LeBreton (5), Lande, Derville, and Godeau (6), Carlson *et al.* (7), and others. However, only meager information exists regarding the time needed to reach equilibrium, any lags which may occur locally during the transportation period, and the rôle of the alimentary tract in the storage of alcohol.

The rate at which various parts of the body attain *storage equilibrium* for alcohol is of interest not only scientifically but also from a medicolegal standpoint, since definite knowledge of this subject will settle whether it is possible to predict brain alcohol by analysis of blood, spinal fluid, liver, muscle, etc. Gettler and Freireich (8) have claimed that spinal fluid alcohol more closely parallels brain alcohol than does blood alcohol, although most of their figures for the blood-brain alcohol ratio are fairly uniform and within the limits found by other investigators. In the matter of spinal fluid, Mehrtens and Newmann (9) and Fleming and Stotz (10) have reported a considerable lag in lumbar spinal fluid alcohol as compared with blood alcohol following small doses of alcohol to human beings. For this reason Mehrtens and Newmann (9) contend that lumbar spinal fluid is less satisfactory than blood for use in predicting brain alcohol.

The rate of absorption of orally administered alcohol has been estimated by two procedures. The first is to follow the blood alcohol concentration and to assume that the peak in this curve represents the point of complete absorption (Mellanby (11), Widmark (12), Jungmichel (13)). This method is open to the criticism that a lag in the storage of alcohol in various parts of the body may cause the peak in blood alcohol to appear long before absorption is complete, or perhaps may produce no peak at all but the Grehant "plateau" (14). Haggard and Greenberg (15) have recently claimed that a comparison of the curves for blood alcohol, after oral administration, with those following intravenous administration shows that about 6 hours are required for complete absorption of alcohol given by mouth. This figure is much higher than has been estimated by other workers using blood alcohol data. More direct evidence regarding the rate of absorption of alcohol from the alimentary tract has been obtained by Nemser (16) and Hanzlik and Collins (17) in noting the rate of disappearance of alcohol from sections of the alimentary canal, and by Voltz and Dietrich (18) and Cori, Villiaume, and Cori (19) by determining residual alcohol in the entire alimentary canal and contents of animals sacrificed after receiving alcohol by mouth. We have recently shown (20) that in these more direct studies a correction should be applied for alcohol stored in the alimentary tract owing to equilibrium with the rest of the body; otherwise, the period for so called complete absorption will equal the time required for the disappearance of alcohol from the body.

The effect of tissue water on the storage of alcohol has been studied by Nicloux (21) who immersed fish in dilute alcohol solutions and found that the alcohol concentration per unit of water in the muscles and total bodies of the fish reached 0.85 to 0.95 of the concentration in the exterior fluid.

In this paper we report studies of the distribution of alcohol in the bodies of a large number of dogs, groups of which were killed at various intervals following oral administration of alcohol. Data were thus obtained both on the velocity of alcohol distribution to different tissues and on the final tissue concentration ratios as equilibrium was approached. Other groups of dogs received alcohol intravenously, in order to test whether the same gastrointestinal concentration of alcohol resulted when approached from below as when approached from above by oral administration of the same dose of alcohol. The question of correlating tissue water and alcohol content was studied by making moisture determinations on the tissues analyzed for alcohol in the cases of a number of dogs which had reached equilibrium in the storage of alcohol. We also include in this paper results of alcohol analyses made upon blood and spinal fluid of forty-six human subjects.

EXPERIMENTAL

Dog Studies—The dogs used for these experiments had received water but no food for a period of 12 hours prior to the administration of alcohol. In the oral experiments the alcohol was given by stomach tube in a concentration of 24 per cent by volume. (With the dogs receiving 6.0 gm. per kilo, the concentration was 32 per cent.) Following this the dogs were kept quiet by an attendant and were carefully watched for a period of 2 hours, or until they were killed, if the time interval was less than 2 hours. In those dogs in which vomiting occurred, in spite of careful attention, the animals were not killed but were reserved and used some days later. At the end of the specified time intervals the dogs were quickly killed by a blow on the head, after which autopsy was performed as rapidly as possible and the materials to be examined were immediately placed in separate containers, cooled in a refrigerator, and analyzed for alcohol during the next few hours. In most of the experiments the stomach and contents, if any, were analyzed separately, but with a few of the dogs they were analyzed together. The intestine was divided about midway between the pylorus and the anus and these two portions together with their contents were analyzed separately. In a few of the experiments the intestine was not divided prior to the analyses. With brain, the entire organ was taken. Muscle specimens were removed from around the femur. The blood specimens were drawn from the saphenous vein and were preserved with sodium fluoride. Alcohol determinations were made by a microdichromate method recently described by one of us (R.N.H. (22)). The time intervals between the administration of alcohol and the death of the dogs varied from 15 minutes to 12 hours, and the dosage of alcohol varied from 0.5 gm. per kilo to 6.0 gm. per kilo, although most of the animals received 3.0 gm. per kilo.

In the intravenous experiments eight dogs received 3.0 gm. of alcohol per kilo and four were killed at the end of 2 hours and four at the end of 3 hours. The alcohol was administered in the form of a 12 to 18 per cent solution in 0.9 per cent sodium chloride. The injection was made into the saphenous vein and required from 30 to 50 minutes. The intervals recorded are from the time when the injection was started.

Human Studies—The forty-six subjects used for these analyses were patients admitted to the emergency clinic of the Indianapolis City Hospital. Most of them showed evidence of having consumed alcohol. The blood was drawn from the cubital vein of the arm and the spinal fluid from the lumbar region of the spine, both specimens being taken within a period of 5 minutes. Care was taken to avoid any contamination with alcohol at the sterilized areas where the skin was punctured. All specimens were preserved with sodium fluoride and were analyzed promptly. No attempt was made to determine the time interval following the last ingestion of alcohol. In eight of the subjects, further specimens were obtained after some hours.

Results

Dog Studies—The concentrations of alcohol in various parts of the alimentary tract and in the blood, at an interval of 2 hours or 3 hours after intravenous administration of 3.0 gm. of alcohol per kilo, are shown graphically in Fig. 1. For purposes of comparison Fig. 1 also records similar data obtained after intervals of 15 minutes, 2 hours, 3 hours, and 12 hours following oral administration of the same dose of alcohol. It will be noted that after intravenous administration the concentration of alcohol along the alimentary tract is much the same at the end of 2 hours as at the end of 3 hours, indicating that these time intervals are probably sufficient for attaining equilibrium. While the dogs sacrificed 15 minutes following alcohol by stomach tube showed very high levels of alcohol in the stomach and stomach contents and rather low levels in the intestine, the animals killed at periods of 2 hours and 3 hours following oral administration had almost the same concentrations of alcohol as were obtained from the dogs receiving the same dose of alcohol intravenously. Although a few of the dogs killed at the end of 2 or 3 hours following oral

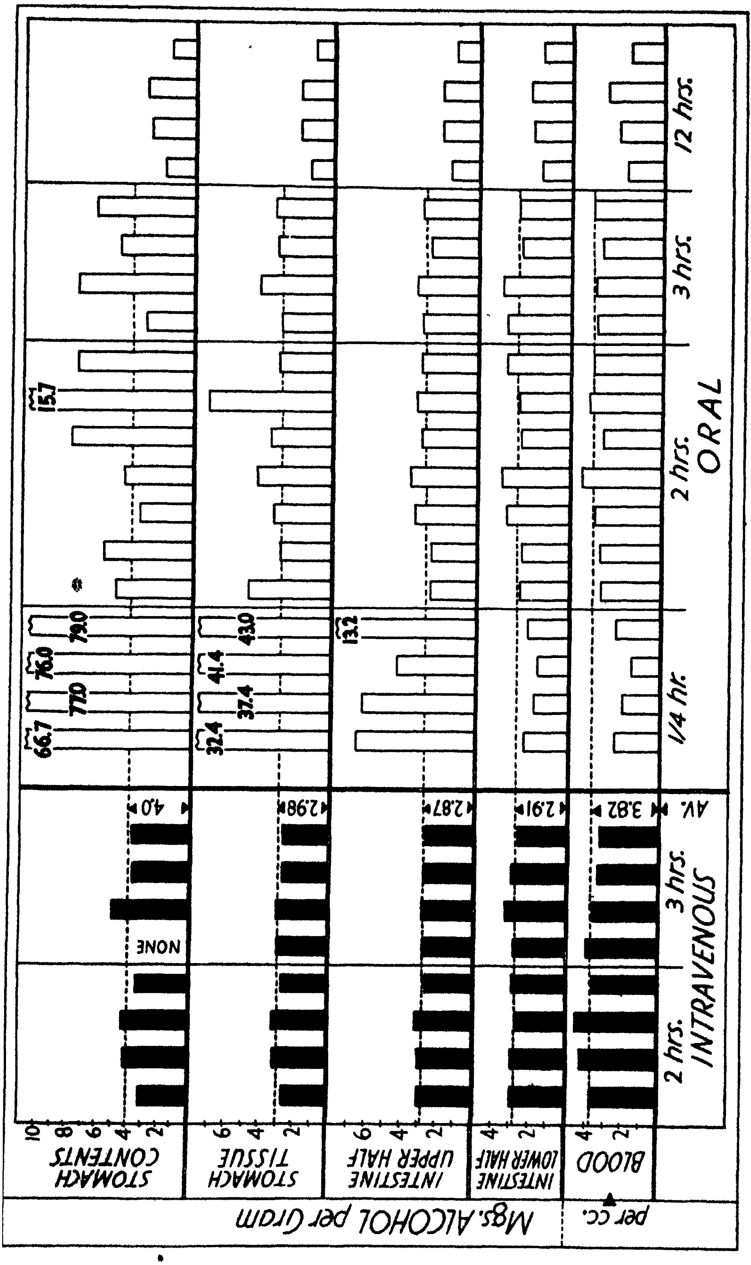

FIG. 1. Alcohol levels in the alimentary tract and blood of twenty-seven dogs killed at certain intervals following oral or intravenous administration of 3.0 gm. of alcohol per kilo. The data in each vertical column are from a single animal.

administration had not quite reached equilibrium as regards the stomach contents and stomach tissue, a comparison of these results with those obtained at the end of 15 minutes will show that even these animals had almost reached equilibrium. Intravenous and oral administration resulted in practically the same average concentration of blood alcohol at the end of 2 hours or 3 hours. The group of dogs receiving alcohol orally and killed at the end of 12 hours still exhibited alcohol along the gastrointestinal tract and the ratios of these figures to the blood alcohol for this time interval are much the same as those observed at the end of 2 hours or 3 hours. The results with the dogs killed after 12 hours indicate considerable differences among these animals in the rapidity with which they were able to burn alcohol. In the intravenous experiments the percentages of administered alcohol stored in the entire gastrointestinal tract were 4.31, 6.52, 6.22, and 5.10 for the animals killed at the end of 2 hours and 3.73, 5.54, 4.14, and 6.12 for the animals killed at the end of 3 hours, the average for all eight animals being 5.21 per cent.

TABLE I

Extent of Absorption of Orally Administered Alcohol after Various Time Intervals

Alcohol administered	Time interval	Unabsorbed alcohol (excess above equilibrium values)		Average absorbed alcohol
		Individual results	Average	
gm. per kg.	*hrs.*	*per cent of administered alcohol*	*per cent*	*per cent*
3.0	¼	54.6, 33.4, 55.3, 47.6	47.7	52.3
3.0	½	50.0, 53.4, 34.7, 32.0	42.5	57.5
3.0	1	9.9, 0.8, 1.6, 27.5, 3.6, 23.3, 4.0	10.1	89.9
3.0	1½	7.9, 8.3, 1.3, 9.6	6.8	93.2
3.0	2	0.9, 1.7, 0.7, 9.6, 0.9, 9.5, 1.0	3.5	96.5
3.0	3	0.3, 2.9, 0.3, 0.5	1.0	99.0
0.5	½	6.2, 15.2, 2.4, 1.1	6.2	93.8
6.0	½	54.6, 40.1, 52.7	49.1	50.9
1.0	1	3.0, 11.2, 7.2, 2.8	6.1	93.9

Table I records the extent of absorption of orally administered alcohol after various time intervals, calculated as excess above equilibrium values. For the animals receiving 3.0 gm. of alcohol per kilo, the equilibrium values employed were the average alcohol concentrations resulting in the alimentary tract of the dogs receiving this dose of alcohol by vein and killed at the end of 2 or 3 hours. For other dosages of alcohol proportional equilibrium figures were assumed. Decreasing the dose administered per kilo from 3.0 gm. to 1 or 0.5 gm. caused a marked rise in the per cent absorbed in the same period of time, while increasing the dose to 6.0 gm. per kilo resulted in a fall in the per cent absorbed during the ½ hour period used. Most of the "unabsorbed" alcohol was found in the stomach and contents, with very little in the intestine, particularly the lower half. In order to conserve

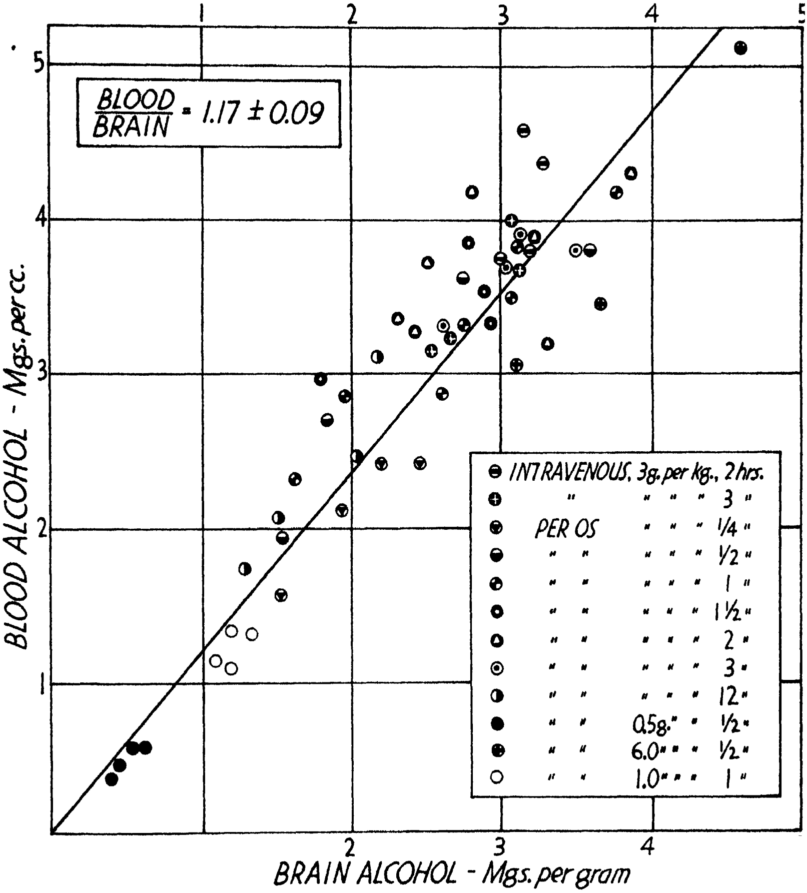

FIG. 2. Correlation between brain alcohol and blood alcohol in 53 dogs

space we have omitted from Table I detailed figures regarding weights and alcohol concentrations of various parts of the alimentary tract. These details were given for a few experiments in our preliminary publication (20).

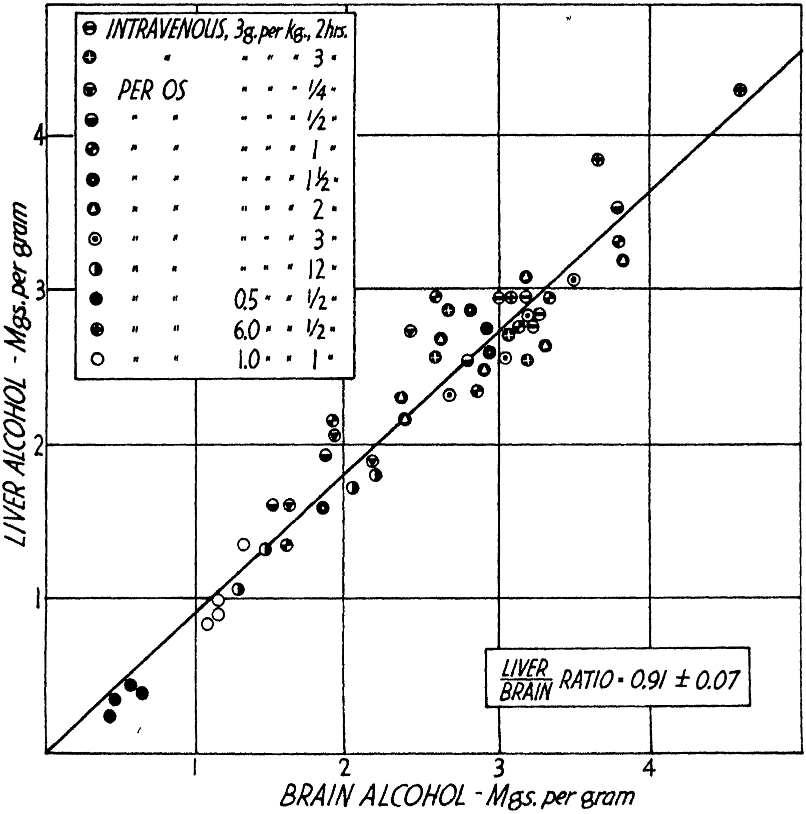

FIG. 3. Correlation between brain alcohol and liver alcohol in 53 dogs

Figs. 2 to 4 record the concentrations of alcohol found in the blood, liver, muscle, and brain and demonstrate a close parallelism between the alcohol concentrations of the brain, blood, and liver. With muscle there was a distinct lag in the concentration of alcohol for at least 1 hour, although reducing the dose to 0.5 gm. per kilo resulted in figures close to equilibrium at the end of ½ hour.

Unfortunately, no muscle specimens were taken for the interval between 1 and 3 hours. Muscle specimens for all dogs killed at the end of 3 hours or later were found to have reached equilibrium with the other organs in the storage of alcohol.

Table II gives the per cent of water in the organs studied from twelve of the dogs and the concentrations of alcohol per unit of water in these organs. These results indicate that, when equilibrium is reached, brain, liver, muscle, stomach tissue, upper intestine, and lower intestine store practically the same amount of alcohol per gm. of water, while blood and stomach contents uniformly store somewhat more alcohol on this basis. The figures for alcohol per gm. of blood given in Table II were calculated from our figures per cc., assuming an average specific gravity of 1.054

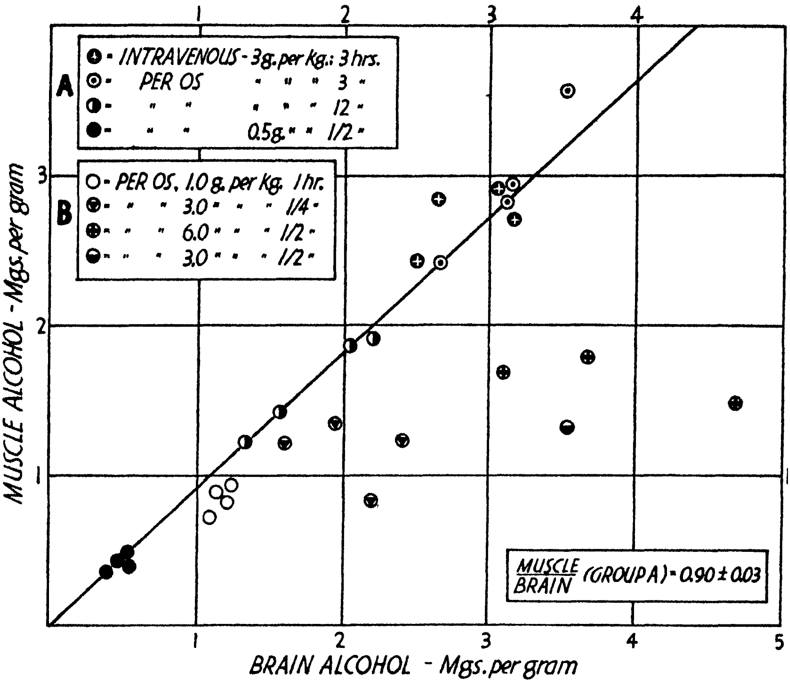

FIG. 4. Correlation between brain alcohol and muscle alcohol in twenty-eight dogs. The line represents the average of the ratios of the dogs which had reached equilibrium (Group A).

TABLE II

Influence of Tissue Water on Alcohol Storage Equilibrium Following 3 Gm. of Alcohol per Kilo

Dog No.	1	2	3	4	5	6	7	8	9	10	11	12	Average
Method of administration	Vein	Vein	Vein	Vein	Vein	Oral	Oral	Oral	Oral	Oral	Oral	Oral	
Time interval, *hrs*	2	2	3	3	3	3	3	3	12	12	12	12	
Water content, *per cent*													
Brain	76.8	74.1	76.5	76.7	77.1	76.6	74.4	77.9					76.3
Blood	76.8	76.6	75.8	79.9	79.7	78.9	79.2	82.0					78.6
Liver	72.3	72.7	75.9	70.2	74.1	73.8		73.4					73.2
Muscle			73.3	65.7	69.1	70.0	72.2	73.1					70.6
Stomach contents	97.0	96.7		98.9	80.4	95.1	98.2	80.9					92.5
" tissue	80.3	79.2	80.6	80.0	78.0	78.3	82.1	80.9					79.9
Intestine, upper half	73.6	71.4	76.4	76.5	75.4	76.1	78.6	77.6					75.7
" lower "	75.1	74.4	76.8	78.8	78.9	76.8	79.5	78.6					77.4
Alcohol per gm. water, *mg.*													
Brain	4.22	4.25	3.96	4.09	3.53	4.01	4.70	3.46	1.95*	2.68*	2.91*	1.72*	
Blood	5.35	5.60	5.01	4.44	3.88	4.50	4.55	3.85	2.44*	2.93*	3.75*	2.16*	
Liver	3.91	4.04	3.59	3.78	3.82	3.73		3.16	1.84*	2.36*	2.91*	1.72*	
Muscle			3.86	4.11	3.99	3.96	4.91	3.28	1.87*	2.59*	2.67*	2.67*	
Stomach contents	4.30	4.57		5.10	4.58	3.27	7.64	6.04	2.14*	3.14*	3.36*	1.64*	
" tissue	4.14	4.25	3.68	2.93	3.60	3.97	5.34	4.23	1.80*	2.47*	2.54*	1.43*	
Intestine, upper half	4.10	4.50	3.48	3.65	3.60	4.08	4.14	3.37	1.91*	2.61*	2.65*	1.86*	
" lower "	3.91	3.67	3.65	4.21	3.81	4.26	4.55	3.28	1.91*	2.61*	2.65*	1.86*	
Ratios													
Blood-brain	1.27	1.32	1.27	1.09	1.10	1.12	0.97	1.11	1.25	1.09	1.29	1.25	1.18 ±0.08
Liver-brain	0.91	0.95	0.98	0.96	1.08	0.93		0.91	0.95	0.88	0.89	0.86	0.94 ±0.04
Muscle-brain			0.98	1.04	1.13	0.99	1.04	0.95	0.96	0.97	0.92	0.97	1.01 ±0.04

Ratios—*concluded*													
Stomach contents-brain......	1.00	1.07		1.28	1.30		†	†	1.10	1.17	1.15	0.95	1.13 ±0.08
" tissue-brain.......	0.96	1.00	1.01	0.74	1.02	0.99	†	†	0.92	0.92	0.87	0.83	0 93 ±0.06
Upper intestine-brain........	0.95	1.06	0.95	0.92	1.02	1.02	0.88	0.97	0.98	0.97	0.93	0.93	0.97 ±0.03
Lower intestine-brain........	0.91	0 86	1.00	1.06	1 08	1.06	0.97	0.95	0.98	0.98	0.90	1.08	0.99 ±0.05

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* Calculated, assuming average figures for water content of organs from Dogs 1 to 8.

† Equilibrium not yet attained.

for dog blood, as given by Austin, Cullen, Gram, and Robinson (23).

Human Studies—Fig. 5 shows an average ratio of spinal fluid alcohol-blood alcohol of 1.18 ± 0.09.[1] When this ratio is recalculated on the basis of water content of the fluids, assuming that average human blood contains 0.834 gm. of water per cc. (24) and average spinal fluid 0.99 gm. of water per cc. (25), this gives a ratio of spinal fluid alcohol-blood alcohol of 0.996. Table III records the changes observed after certain intervals of time in the blood and spinal fluid alcohol of eight of these subjects.

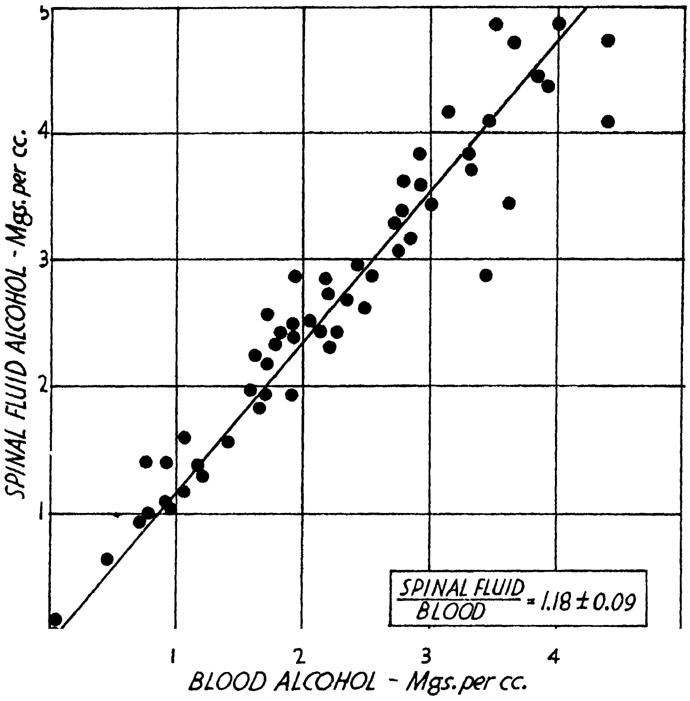

Fig. 5. Correlation between blood alcohol and spinal fluid alcohol in forty-six human subjects.

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[1] Wherever ± is used in this paper, it refers to the probable error.

TABLE III

Changes in Blood and Spinal Fluid Alcohol of Human Subjects after Various Intervals of Time

Subject No.	Time after first specimen	Blood alcohol	Spinal fluid alcohol	Spinal fluid alcohol / Blood alcohol
	hrs.	*mg. per cc.*	*mg. per cc.*	
39		2.13	2.60	1.22
	2	1.76	2.28	1.30
	11	0	0	
40		0.48	0.61	1.27
	3¼	0	0	
41		4.39	4.52	1.04
	2	3.55	4.84	1.36
	10	1.07	1.58	1.47
42		3.28	3.73	1.14
	3	2.92	3.76	1.29
43		3.48	2.90	0.83
	2	3.00	3.44	1.15
44		2.82	3.60	1.28
	4	1.81	2.34	1.29
	8	0	0	
45		1.63	1.98	1.22
	3½	1.02	1.17	1.15
46		3.10	4.13	1.33
	2½	2.86	3.17	1.11

DISCUSSION

Vollmering in 1912 (26) suggested that muscle alcohol lags behind blood alcohol during the first 1 or 2 hours following alcohol administration, but his experimental evidence on this point is very limited and somewhat contradictory. He also claimed that brain alcohol lags far behind muscle alcohol. The very definite lag in muscle alcohol, which we found during the periods of short duration, may have some importance in explaining considerable fluctuations in blood alcohol frequently seen during the first 2 hours following oral administration. It might even account for some differences in "tolerance" to alcohol, since the muscles constitute a large fraction of the total body weight, and an unusual lag in storage of alcohol in the muscles would result in higher initial concentrations of alcohol in the blood and in organs having better circulation, such as the brain. Whether this lag in muscle alcohol will hold for skeletal muscles other than those of the thigh can only be determined by further experiments.

The blood-brain ratios for periods shorter than 1 hour are slightly decreased below the general average, especially with the higher doses of alcohol. This is probably explained by the fact that the blood was drawn from the saphenous vein and that, owing to the delay of alcohol storage in muscle, blood circulating through the leg would lose more alcohol than the blood from the brain. Later we plan to compare the alcohol content of blood from various parts of the circulatory system.

The higher relative concentrations of alcohol per gm. of water which we found in the blood, spinal fluid, and stomach contents would seem to lend support to Nicloux's hypothesis of "bound" water in the more solid tissues of the body (21).

The rather constant ratio which we observed for spinal fluid-brain alcohol in our human subjects should not be interpreted as contradicting the findings of Mehrtens and Newmann (9), since probably at least ½ hour had elapsed between the last ingestion of alcohol and the collection of the specimens. The rise in this ratio which we observed in Cases 41, 42, and 43 suggests a lag in spinal fluid alcohol.

Since our animals had received no food for some time prior to the administration of alcohol, the rate of absorption of alcohol from the gastrointestinal tract was undoubtedly somewhat higher than would have been observed had food been administered near the time when the alcohol was given.

Contrary to the claim of Gettler and Freireich (8) our results with dogs demonstrate that brain alcohol may at all times be predicted with reasonable accuracy from the alcohol content of blood, and our figures for human beings indicate that in the practical testing of human subjects the alcohol concentrations of blood and spinal fluid run so nearly parallel that spinal fluid specimens are certainly not to be preferred to blood for this purpose.

SUMMARY

1. Determinations were made of the distribution of alcohol in various parts of the bodies of 53 dogs which had received alcohol orally or intravenously and were killed after certain time intervals.

2. Where the period was 2 hours or 3 hours, intravenous and oral administration of 3 gm. of alcohol per kilo resulted in practically the same concentration ratios of alcohol in blood, stomach contents, stomach tissue, upper intestine, and lower intestine, about 5 per cent of the administered alcohol being stored in the alimentary tract. These equilibrium values were still maintained when the period was extended to 12 hours.

3. Following the oral administration of 3 gm. of alcohol per kilo, the unabsorbed alcohol, calculated as excess above equilibrium values for the alimentary tract, decreased from an average of 47.7 per cent at 15 minutes to 1.0 per cent at 3 hours. With 0.5 and 6.0 gm. per kilo, the unabsorbed alcohol at ½ hour averaged 6.2 and 49.1 per cent respectively.

4. Although the time intervals varied from 15 minutes to 12 hours, no changes were observed in the concentration relations of alcohol in blood, brain, and liver. The average ratios of alcohol concentrations found in the 53 dogs were (brain = 1), blood 1.17 ± 0.09, and liver 0.91 ± 0.07. With muscle there was a distinct lag during the 1st hour but equilibrium was attained within 3 hours, after which the muscle-brain ratio was 0.90 ± 0.03.

5. After equilibrium resulted, alcohol was stored in about the same proportion as the water content of the materials analyzed. The average ratios on this basis were (brain = 1), blood 1.18 ± 0.08, liver 0.94 ± 0.04, muscle 1.01 ± 0.04, stomach contents 1.13 ± 0.08, stomach tissue 0.93 ± 0.06, upper intestine 0.97 ± 0.03, and lower intestine 0.99 ± 0.05.

6. The spinal fluid-blood alcohol ratios of forty-six human subjects averaged 1.18 ± 0.09. On the basis of average water content in these fluids, the recalculated alcohol ratio was spinal fluid-blood = 0.996.

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BIBLIOGRAPHY

1. Grehant, N., *Compt. rend. Acad.*, **120**, 1154 (1895); **123**, 192 (1896); **129**, 746 (1899); *Compt. rend. Soc. biol.*, **48**, 839 (1896); **51**. 808 (1899); **52**,

894 (1900); **55**, 225, 376, 802, 1264 (1903); *J. physiol. et path. gén.*, **9**, 879 (1907).

2. Nicloux, M., *Compt. rend. Soc. biol.*, **51**, 980 (1899); **107**, 997 (1931).
3. Mellanby, E., *Med. Research Council, Special Rep. Series, No. 31* (1919).
4. Carpenter, T. M., *J. Pharmacol. and Exp. Therap.*, **37**, 217 (1929).
5. LeBreton, E., *Compt. rend. Soc. biol.*, **117**, 704 (1934).
6. Lande, P., Derville, P., and Godeau, J., *Ann. méd. légale*, **17**, 11 (1937).
7. Carlson, A. J., Kleitman, N., Muehlberger, C. W., McLean, F. C., Gullicksen, H., and Carlson, R. B., Studies on the possible intoxicating action of 3.2 per cent beer, Chicago, 42 (1934).
8. Gettler, A. O., and Freireich, A. W., *J. Biol. Chem.*, **92**, 199 (1931); *Am. J. Surgery*, **27**, 328 (1935).
9. Mehrtens, H. G., and Newmann, H. W., *Arch. Neurol. and Psychiat.*, **30**, 1092 (1933).
10. Fleming, R., and Stotz, E., *Arch. Neurol. and Psychiat.*, **33**, 492 (1935).
11. Mellanby, E., *Brit. J. Inebriety*, **17**, 157 (1920).
12. Widmark, E. M. P., Die theoretischen Grundlagen und die praktische Verwendbarkeit der gerichtlich-medizinischen Alkoholbestimmung, Berlin (1932).
13. Jungmichel, G., Alkoholbestimmung im Blut, Berlin (1933).
14. Grehant, N., *Compt. rend. Soc. biol.*, **51**, 946 (1899).
15. Haggard, H. W., and Greenberg, L. A., *J. Pharmacol. and Exp. Therap.*, **52**, 167 (1934).
16. Nemser, M. H., *Z. physiol. Chem.*, **53**, 356 (1907).
17. Hanzlik, P. J., and Collins, R. J., *J. Pharmacol. and Exp. Therap.*, **5**, 185 (1913).
18. Voltz, W., and Dietrich, W., *Biochem. Z.*, **68**, 118 (1915).
19. Cori, C. F., Villiaume, E. L., and Cori, G. T., *J. Biol. Chem.*, **87**, 19 (1930).
20. Harger, R. N., and Hulpieu, H. R., *Proc. Soc. Exp. Biol. and Med.*, **32**, 1247 (1935).
21. Nicloux, M., *Bull. Soc. chim. biol.*, **16**, 822 (1934).
22. Harger, R. N., *J. Lab. and Clin. Med.*, **20**, 746 (1935).
23. Austin, J. H., Cullen, G. E., Gram, H. C., and Robinson, H. W., *J. Biol. Chem.*, **61**, 833 (1924).
24. Bodansky, M., Introduction to physiological chemistry, New York, 3rd edition, 218 (1934).
25. Levinson, A., Cerebrospinal fluid, St. Louis, 126 (1929).
26. Vollmering, J., Inaugural dissertation, Giessen (1912); see Heffter, A., Handbuch der experimentellen Pharmakologie, Berlin, **1**, 282 (1923).

THE PHENOL AND IMIDAZOLE CONTENT OF THE BLOOD

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In most investigations of phenol content of blood colorimetric methods have been applied directly to blood filtrates. Little if any effort has been made to separate the more truly phenolic, ether-soluble compounds (phenol, *p*-cresol, diphenols, aromatic hydroxy acids, etc.) from the ether-insoluble nitrogenous constituents (imidazoles, purines, indole, amino acids, etc.). Thus the generally reported values for "blood phenols" really represent the diazochromogenic resultants of groups of chemically different substances rather than an individual compound or even a group of similar compounds.

Following the early work of Baumann (1) and his contemporaries (2), Folin and Denis (3) in 1915 described a quantitative method for determining urinary and blood phenols (4–17) and in 1924 Theis and Benedict (18) employed the *p*-nitroaniline diazotization reaction of Moir (19–22). Recently Lambrechts and Barac (23) concluded that this reaction given by blood filtrates is due mainly to imidazoles (24–26). These phenolic and nitrogenous compounds have been studied, also, by means of the Pauly diazosulfanilic acid reaction (27–36), Millon's reaction (30), and the xanthoproteic reaction (37 47). Becher *et al.* (37) found that ether-soluble phenols of normal human blood are negligible by this latter method but tend to increase in uremia. Likewise "blood phenols" have been reported to be elevated in severe kidney and liver disease by the *p*-nitroaniline method (48-50).

In the course of experiments involving the ether extraction of phenols from urine (51), it occurred to us that in the accepted colorimetric methods for determining blood phenols (7, 18, 52), since they are carried out directly on the blood filtrates, reagents are employed which might react largely with ether-insoluble and therefore presumably non-phenolic compounds. A preliminary experiment confirmed our suspicion. Therefore we decided to develop an ether extraction method to measure more accurately the free phenol content of the blood. In addition, we made a comparative examination of the phenol and imidazole content of the blood, the "diazo value," using several colorimetric methods both before and after ether extraction. This innovation separates the chromogens into two fractions—the more truly phenolic compounds which pass into the ether layer, and the other reactive compounds which remain in the ether-extracted filtrates.

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EXPERIMENTAL

The following methods were used, with certain modifications, for the direct determination of the "diazo value" of the blood filtrates: (1) the Theis-Benedict (18) application of the Moir (19) *p*-nitroaniline reaction; (2) a modified Pauly (27) Koessler and Hanke (29) sulfanilic acid reaction; and (3) an adaptation of Meschkowa's (36) modified Pauly reaction.

Apparatus and Materials—After the routine blood chemistry determinations in the hospital laboratory were completed, the residual oxalated bloods were pooled and a 1:5 tungstic acid blood filtrate was prepared according to the usual Folin-Wu technique. 60 cc. of the filtrate, equivalent to 12 cc. of blood, were transferred to a modified Kutscher-Steudel all-glass extraction apparatus (Fig. 1) designed by one of us (H.B.W.). A detailed description of this apparatus will be published elsewhere.

It was necessary to eliminate all corks and rubber connections, since they persistently yield phenolic or chromogenic substances which react with the diazo reagents. Likewise it was noted that ether, upon standing in glass bottles, gradually develops non-volatile substances, perhaps peroxides, which react with diazotized *p*-nitroaniline. However, these substances are not present in fresh ether (United States Pharmacopœia X, Merck).

Theis-Benedict p-Nitroaniline Method—This well known method for "blood phenols" was used essentially as outlined by Theis and

Benedict (18). However, it was noted that a histidine standard containing 0.028 mg. of N also gives an orange-red color with diazotized *p*-nitroaniline which matches the color given by the blood filtrates much better. It seems more logical to use an amino acid as a standard, since our data emphasize the fact that normal blood contains practically no true phenols—the diazo-reactive compounds apparently being nitrogenous in character. Nevertheless we report the "diazo value" of the blood filtrates both as phenol and as histidine.

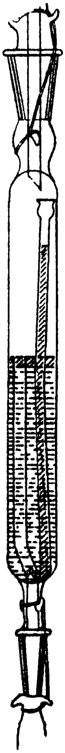

FIG. 1. Extraction apparatus

In order to obtain uniformity all colorimetric readings were made exactly 3 minutes after the addition of the sodium carbonate. Both phenol and histidine solutions of varied concentrations follow Beer's law well when treated with diazotized *p*-nitroaniline and sodium carbonate, but this proportionality is lessened slightly by the presence of sodium acetate and gum arabic. Hence proportionality curves were plotted from the data given in Table I and all values for the blood filtrates were obtained from these curves. Fortunately, varying quantities of blood filtrate diluted with water seem to show similar deviation from true proportionality.

A stock histidine solution was prepared by dissolving 0.1644 gm. of *l*-histidine monohydrochloride (Hoffmann La Roche) in distilled water and diluting to 250.50 cc. The stock solution keeps for at least 6 months when in the cold, but the various standards tend to deteriorate, especially at room temperature. The stock phenol solution containing 2 gm. of phenol per 2000 cc. of 0.1 N HCl was checked by iodometric titration. In the cold it remains stable for at least a year—the standards for a month or longer. The standards containing 0.028 mg. of histidine nitrogen and 0.025 mg. of phenol per 10 cc. of solution were used in the Theis-Benedict method.

TABLE I

Proportionality Curves Given by Phenol and Histidine with p-Nitroaniline Reagents

Phenol determination					Histidine determination				
Phenol added	Colorimeter reading	Phenol found		Color match	Histidine added	Colorimeter reading	Histidine found		Color match
mg. per 10 cc.	*mm.*	*mg. per 10 cc.*	*per cent*		*mg. per 10 cc.*	*mm.*	*mg. per 10 cc.*	*per cent*	
0.0250*	20.0*				0.0280*	20.0*			
0.0125	34.0	0.0147	117.6	Fair	0.0140	36.5	0.01534	109.6	Fair
0.0150	29.0	0.0172	114.9	Good	0.0196	29.3	0.01911	97.5	Good
0.0200	24.1	0.0208	104.0	"	0.0224	26.0	0.02154	96.2	"
0.0225	22.2	0.0225	100.0	"	0.0252	22.8	0.02456	97.5	"
0.0250	20.0	0.0250	100.0	"	0.0280	20.2	0.02772	99.0	"
0.0300	17.1	0.0292	97.3	"	0.0336	17.0	0.03294	98.0	"
0.0400	13.3	0.0376	94.0	"	0.0448	13.0	0.04308	96.2	"
0.0500	11.1	0.0451	90.2	Fair	0.0560	10.7	0.05234	93.5	Fair

* Standard set at 20.0 mm. in the colorimeter.

Modified Pauly (27) Koessler and Hanke Sulfanilic Acid Reaction—We have attempted, also, to develop other methods for the determination of the "diazo value" of the blood by substituting

TABLE II

Proportionality Curves Given by Phenol and Histidine with Diazobenzenesulfonic Acid Reagents

By modified Koessler and Hanke procedure								Modified Meschkowa procedure			
Phenol determination				Histidine determination				Histidine determination			
Phenol added	Phenol recovered		Color match	Histidine N added	Histidine N recovered		Color match	Histidine N added	Histidine N recovered		Color match
mg. per 10 cc.	*mg. per 10 cc.*	*per cent*		*mg. per 10 cc.*	*mg. per 10 cc.*	*per cent*		*mg. per 10 cc.*	*mg. per 10 cc.*	*per cent*	
0.01250*				0.0070*				0.0140*			
0.00625	0.00641	102.6	Good	0.0028	0.00233	83.2	Yellower	0.0070	0.0070	100.0	Good
0.00875	0.00833	95.2	"	0.0042	0.00389	92.6	Fair	0.0084	0.00816	97.1	"
0.01000	0.00976	97.6	"	0.0056	0.00510	91.1	Good	0.0112	0.01098	98.0	"
0.01125	0.01087	96.6	"	0.0070	0.00710	101.4	"	0.0140	0.01411	100.8	"
0.01250	0.01259	100.7	"	0.0084	0.00903	107.5	Fair	0.0168	0.01618	96.3	"
0.01500	0.01405	93.7	"	0.0098	0.01170	119.4	Redder	0.0224	0.02188	97.7	"
0.02000	0.02080	104.0	"	0.0112	0.02800	250.0	"	0.0280	0.02850	101.8	"
0.02500	0.02381	95.2	"								

* Standard set at 20.0 mm in the colorimeter.

sulfanilic acid for the *p*-nitroaniline. In this manner like volumes of solutions are used; *i.e.*, 10 cc. of blood filtrate, 10 cc. of the histidine standard containing 0.007 mg. of nitrogen, and 10 cc. (0.0125 mg.) of the phenol standard. To each tube are added 2 cc, of a freshly prepared solution of diazotized sulfanilic acid (29, 36). The contents of the tubes are mixed and 1 minute later 2 cc. of 1.1 per cent sodium carbonate are added. The color comparison is made at the end of 5 minutes. The various solutions develop a straw-yellow color and match fairly well in the colorimeter. Upon standing there is no evidence of any turbidity, although the histidine standard tends to develop a red tinge, hence all readings are made exactly 5 minutes after the addition of the sodium carbonate. It is obvious from the figures in Table II that only in the case of histidine need proportionality curves be used.

Adaptation of the Meschkowa Modification of the Pauly Reaction—Recently, Meschkowa (36) has described an interesting modification of the Pauly (27) diazo reaction for carnosine. In making use of this innovation we add 10 cc. of freshly diazotized sulfanilic acid to 10 cc. of the blood filtrate and to 10 cc. of the histidine standard containing 0.014 mg. of nitrogen. After 3 hours 2 cc. of 20 per cent sodium carbonate are added to each tube and the filtrates are read against the standard 15 minutes later. The color of the solutions is deeply intensified as compared to that produced by the sulfanilic acid reagents in the older methods in which alkalization is carried out immediately. When one looks downward through the solutions both standard histidine and filtrate appear a deep rose-red. However, the filtrate has a definite yellow tinge when examined in the colorimeter. Thus, only a fair comparison can be obtained. The blood "diazo value" is calculated directly from the colorimetric reading, since histidine shows good proportionality under these experimental conditions (Table II). Phenol solutions show no color intensification when subjected to this procedure—the solutions remaining light yellow as usual. Because phenol cannot be used as a standard, our values by this method are reported as histidine only.

Influence of Ether Extraction on "Diazo Value" of Blood—The three methods just described for the determination of the "diazo value" of the blood were now applied directly to a series of blood filtrates both before and after ether extraction. The ether-extracted blood filtrate remaining in the glass extractor was transferred to a flask and the ether volatilized by immersion in hot water (below 85°). Since the blood filtrates are slightly acid (pH 4.8 to 5.6), a release or deconjugation of bound chromogenic materials could possibly occur. However, when test-tubes containing 10 cc. portions of a pooled blood filtrate (pH 4.9) were placed in water baths at various temperatures from 50–90° for 15 minutes, then cooled immediately, and volumes adjusted if necessary, coupled and matched against 10 cc. of the same but unheated blood filtrate, no evidence of hydrolysis or deconjugation could be detected with any of the three methods.

The "diazo value" of a separate sample of the same blood filtrate, which had not been extracted with ether, was determined simultaneously with the ether-extracted specimen in order to keep all variables alike. Thus, not only can the original filtrate and the ether-extracted filtrate be matched against the standards, but these two filtrate preparations can be matched against each other in the colorimeter. In this manner the influence of ether extraction on the "diazo value" of the blood filtrates, a factor which has been persistently ignored by workers in this field, can be ascertained readily.

Estimation of Phenols in Ether Extract of Blood Filtrate—Efforts to develop a quantitative method for estimating phenols in the ether extract of the blood filtrates have not been entirely successful. Studies conducted with pure aqueous solutions of phenol generally resulted in an inadequate phenol recovery. Whereas phenol was not lost from warm or even hot water, it seemed to pass off readily with the ether vapors. After considerable experimentation the following procedure was adopted for the determination of phenols ordinarily found in blood and the recovery of added phenol.

1 cc. of an aqueous phenol solution was added to 50 cc. of blood and shaken for 5 minutes, diluted with 99 cc. of water, and the proteins precipitated with 50 cc. of 10 per cent sodium tungstate and 50 cc. of $\frac{2}{3}$ N H_2SO_4. At the same time another sample of the blood was precipitated in a similar manner but without the addition of phenol. Then 60 cc. of each filtrate were extracted rapidly for 2 hours, the receiver containing 7 cc. of ether, 1.5 cc. of water, and a glass bead. The ether was then volatilized by immersing

the tube in hot water, 2.5 cc. of alcohol were added, and the volume made up to 5 cc. with water. If large quantities of phenol had been added to the blood, the extract was first diluted with water before a sample was taken for analysis. The addition of alcohol is desirable, since it clarifies the solution and produces an intensification of color. 2.5 cc. portions of standard phenol solutions (0.0025 to 0.025 mg. of phenol per 2.5 cc.) were measured into test-tubes and 2.5 cc. of alcohol and 2.0 cc. of freshly diazotized sulfanilic acid added to each tube, and, after a minute, 1 cc. of 1.1 per cent sodium carbonate. The phenol standards immediately developed their familiar yellow; the preparation from the blood filtrate, a straw-yellow color. The unknown was read against the phenol standard to which it most closely matched. Sulfanilic acid was used as the reagent, since it yields but a slight blank with a 50 per cent alcohol solution, whereas *p*-nitroaniline yields a very large color blank. The solutions remain clear for several hours with little if any change in color. The data in Table III indicate, however, that the phenol recovery by means of our present methods is not entirely satisfactory.

TABLE III

Determination of Phenols (by Ether Extraction) in Human Blood Containing Added Phenol

Phenol originally present in blood	Phenol added	Phenol found (a)	Phenol calculated (b)	Error $\frac{100(a-b)}{b}$
mg per cent	*mg per cent*	*mg per cent*	*mg per cent*	*per cent*
0.0237	0.0100	0.0260	0.0337	-22.9
0.0235	0.0470	0.0573	0.0705	−18.7
0.0200	0.1000	0.0980	0.1200	−18.3
0.0113	0.2000	0.1097	0.2113	−48.1
0.0120	0.3000	0.2404	0.3120	−23.0
0.0267	0.6000	0.3198	0.6267	−48.9

Thus the recovery in phenol varies from 51.1 to 81.77 per cent of that added to the blood. Not only are volatile phenols lost during the volatilization of the ether, but phenol itself is adsorbed to some extent by the precipitated blood proteins (11, 23). These various methods for the determination of the "diazo value" of blood filtrates both before and after ether extraction and of the

Free Phenol Content and "Diazo Value" of Blood before and after Ether Extraction

The values are given in mg. per 100 cc. of blood.

	Free blood phenol	Theis and Benedict procedure						Modified Koessler and Hanke procedure						Modified Meschkowa procedure		
		As histidine			As phenol			As histidine			As phenol			As histidine		
		Before	After	Difference	Before	After	Difference	Before	After	Difference	Before	After	Difference	Before	After	Difference
Maximum.......	0.0320	5.44	5.49	+0.32	1.35	1.35	+0.08	1.38	1.36	+0.10	0.75	0.83	+0.08	2.87	2.81	−0.14
Minimum....	0.0087	3.76	3.97	0.00	0.81	0.85	0.00	1.09	1.08	0.00	0.53	0.52	0.00	2.16	2.20	0.00
Average.......	0.0189	4.633	4.644	+0.011	1.163	1.172	+0.009	1.214	1.215	+0.001	0.616	0 623	+0.007	2.538	2.527	−0.011

phenolic content of the ether extract may still retain certain deficiencies; nevertheless, they are sufficiently accurate to give a fair quantitative idea of the relative amounts of ether-soluble phenols and ether-insoluble nitrogenous, but diazo-reactive compounds, in the blood. Hence an analysis of a series of pooled blood specimens for these constituents seemed worth while.

The residual oxalated bloods remaining after the routine chemistry determinations were completed were pooled and used in our experiments. While these mixed blood specimens were in part pathological clinically, they were essentially normal chemically, as judged by occasional blood sugar and non-protein nitrogen determinations. Twenty-three separate blood specimens have been subjected to analysis. The complete data concerning the quantity of ether-soluble phenols and the "diazo value" of the various bloods both before and after ether extraction have been omitted and only the summarized findings are reported in Table IV. We have made no attempt to separate the phenols into volatile and non-volatile fractions nor to estimate the portion present in a conjugated form (generally considered to be negligible).

DISCUSSION

From the summarized data in Table IV it is evident that practically the entire "diazo value" of the blood, generally reported in the literature as phenol, is actually made up of ether-insoluble compounds. Thus the quantity of ether-soluble phenolic compounds, determined by ether extraction methods and calculated as phenol (Table IV, first column) and based on the analysis of twenty-three separate specimens of pooled, human blood, varies from 0.0087 to 0.032 mg. with an average of 0.0189 mg. of phenol per 100 cc. of blood. This is in marked contrast to the values of 1 to 2 mg. of phenol per 100 cc. of blood which have been reported in the literature (18, 20, 22, 47–49, 53) and accepted in the textbooks (52, 54–56) up to the present time. While no claim is made for the accuracy of our values, we feel that they are fairly representative of the actual free phenolic content of human blood. This conclusion is indicated, also, by the fact that when each coupled blood filtrate was set in the colorimeter at 20 mm. and matched against the simultaneously coupled filtrate, which had been previously extracted with ether, no appreciable difference could be detected in the two solutions—either in shade or in intensity of color. Similar conclusions are evident from Table IV which gives the summarized values for the blood filtrates—both before and after ether extraction—when they are coupled and matched against both the histidine and the phenol standards. It is apparent that but a negligible quantity of phenols, quite immeasurable by ordinary colorimetric methods applied directly to the blood filtrates, are removed by ether extraction. Hence it seems obvious, from these observations, that less than 1 per cent of the total "diazo value" of the blood is actually made up of phenols—the reactive substances are practically exclusively ether-insoluble and presumably nitrogenous in nature.

It is pertinent to note that each of the three methods used in our experimental work yields different values for the "histidine" and "phenol" content of the blood. Thus the Theis-Benedict *p*-nitroaniline method gives the highest values, namely 3.76 to 5.44, average 4.633 mg. of histidine, and 0.81 to 1.35, average 1.163 mg. of phenol, per 100 cc. of blood. Our modified Meschkowa procedure gives somewhat lower results, 2.16 to 2.87, average 2.538 mg. of histidine per 100 cc. of blood, whereas our modified Koessler and Hanke method gives even lower values, 1.09 to 1.38, average 1.214 mg. of histidine, and 0.53 to 0.75, average 0.616 mg. of phenol per 100 cc. of blood. Apparently, diazotized *p*-nitroaniline reacts with more compounds in the blood filtrates than do the sulfanilic acid reagents. The difference between the values obtained by the two methods in which the latter reagents were used possibly may be explained by the observations of Kapeller-Adler (32), Fürth *et al.* (30), Jorpes (57), and Meschkowa (36). These workers report that various nitrogen compounds in biological fluids repress the reactivity of diazotized sulfanilic acid and that this inhibiting action can only be overcome by large amounts of reagents which are permitted to react for several hours before the solutions are made alkaline.

We believe that it would make for greater clarity in the interpretation of the biochemistry of the phenols if the various workers would agree uniformly on what the term "phenols" should include and if they would examine the specificity of their analytical methods with this definition in mind. According to our conception "blood phenols" should include only weakly acidic, ether-soluble compounds containing one or more hydroxyl groups attached to an aromatic ring. Other compounds such as tyrosine, which is primarily an amino acid, and imidazoles, purines, indole, skatole, etc., whose only characteristic in common with phenol is the ability to couple with diazonium salts, should be excluded.

SUMMARY

1. Evidence is presented that the generally accepted value for blood phenols (1 to 2 mg. per cent) is too large.

2. A method for estimating the approximate quantity of the ether-soluble phenols of blood has been described which gives a value for normal individuals of about 0.02 mg. per cent.

3. Several methods for determining the "diazo value" of blood have been studied and the results given by these methods are presented and discussed comparatively.

BIBLIOGRAPHY

1. Baumann, E., *Arch. ges. Physiol.*, **13**, 285 (1876).
2. Messinger, J., and Vortmann, G., *Ber. chem. Ges.*, **22**, 2313 (1889).
3. Folin, O., and Denis, W., *J. Biol. Chem.*, **22**, 305 (1915).
4. Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, **36**, 95 (1918).
5. Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, **36**, 99 (1918).
6. Fujiwara, H., and Kataoka, E., *Z. physiol. Chem.*, **216**, 133 (1933).
7. Rakestraw, N. W., *J. Biol. Chem.*, **56**, 109 (1923).
8. Goiffon, R., and Nepveux, F., *Compt. rend. Soc. biol.*, **89**, 1213 (1923).
9. Codounis, A., *Arch. mal. app. digestif.*, **19**, 1078 (1929).
10. Haas, G., and Trautmann, W., *Z. physiol. Chem.*, **127**, 52 (1923).
11. Haas, G., and Schlesinger, E. F., *Arch. exp. Path. u. Pharmakol.*, **104**, 56 (1924).
12. Asada, Y., *Tohoku J. Exp. Med.*, **15**, 363 (1930).
13. Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, **42**, 1678 (1920).
14. Levine, V. E., *Science*, **52**, 612 (1920).
15. Tisdall, F. F., *J. Biol. Chem.*, **44**, 409 (1920).
16. Barac, G., *Rev. belge sc. méd.*, **7**, 669 (1935).
17. Pelkan, K. F., *J. Biol. Chem.*, **50**, 491 (1922). Pelkan, K. F., and Whipple, G. W., *J. Biol. Chem.*, **50**, 499 (1922).
18. Theis, R. C., and Benedict, S. R., *J. Biol. Chem.*, **61**, 67 (1924).
19. Moir, J., *J. South African Inst.*, **5**, 8 (1922).
20. Marenzi, A. D., Éstudios bioquímicos sombre los fenoles, Buenos Aires (1933).
21. Houssay, B. A., *Am. J. Med. Sc.*, **192**, 615 (1936).
22. Falsia, A., *Compt. rend. Soc. biol.*, **111**, 395 (1932).
23. Lambrechts, A., and Barac, G., *Compt. rend. Soc. biol.*, **120**, 522 (1935).
24. Böhm, F., and Grüner, G., *Biochem. Z.*, **287**, 65 (1936).

25. Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharmacol. and Exp. Therap.*, **5**, 275, 611 (1913).

26. Leiter, L. J., *J. Biol. Chem.*, **64**, 125 (1925).

27. Pauly, H., *Z. physiol. Chem.*, **42**, 510 (1904).

28. Weiss, M., and Ssoboliew, N., *Biochem. Z.*, **58**, 119 (1914).

29. Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, **39**, 497 (1919).

30. Fürth, O., and Scholl, R., *Biochem. Z.*, **243**, 274 (1931). Fürth, O., Scholl, R., and Herrmann, H., *Biochem. Z.*, **251**, 148 (1932).

31. Loeper, M., Mougeot, A., and Aubertot, R., *Compt. rend. Soc. biol.*, **116**, 33 (1934).

32. Kapeller-Adler, R., and Boxer, G., *Biochem. Z.*, **285**, 55 (1936).

33. Bussit, J., Recherches analytiques sur l'arginine et l'histidinire, Pas 48 (1935).

34. Trabucchi, E., *Boll. Soc. ital. biol. sper.*, **10**, 264 (1935).

35. Levene, P. A., and Bass, L. W., Nucleic acids, New York, 136 (1931).

36. Meschkowa, N. P., *Z. physiol. Chem.*, **240**, 199 (1936).

37. Becher, E., *Deutsch. Arch. klin. Med.*, **145**, 333 (1924); **148**, 10, 46, 159 (1925). Becher, E., and Koch, F., *Deutsch. Arch. klin. Med.*, **148**, 78 (1925). Becher, E., and Herrmann, E., *Deutsch. Arch. klin. Med.*, **152**, 82 (1926).

38. Musser, J. H., Internal medicine, Philadelphia, 2nd edition, 521 (1934).

39. Rasmussen, H., *Acta med. scand.*, **86**, 302 (1935).

40. Irdelp, N. O., Guchan, M., and Kazim, M., *Presse méd.*, **43**, 1396 (1935).

41. Wuhrmann, F., *Z. klin. Med.*, **127**, 499 (1934).

42. Lefaux, R., *J. pharm. et chim.*, **23**, 437 (1936).

43. Polacios, F. G., *Arch. españ. pediat.*, **19**, 658 (1935).

44. Oefelein, F., *Verhandl. 48 Kong. deutsch. Ges. inn. Med.*, Wiesbaden, 267 (1936).

45. Solaris, C., *Riforma med.*, **52**, 74 (1936).

46. Bonino, M., *J. urol.*, **41**, 236 (1936).

47. Cipriani, C., and Ferrero, A., *Gior. r. Accad. med. Torino*, **33**, 216 (1927).

48. Yanagihara, D., *J. Chosen Med. Assn.*, **24**, 105 (1934).

49. Castex, M. R., and Arnaudo, A. F., *Rev. Assn. méd. argentina*, **49**, 1063 (1935).

50. Castex, M. R., and Arnaudo, A. F., *Prensa méd. argentina*, **23**, 2583 (1936).

51. Schmulovitz, M. J., and Wylie, H. B., *J. Lab. and Clin. Med.*, **21**, 210 (1935).

52. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Interpretations, Baltimore, 514 (1931).

53. Banfi, R. F., Lida, E., and Marenzi, A. D., *Compt. rend. Soc. biol.*, **121**, 358 (1936).

54. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 10th edition, 451 (1931).

55. McClendon, J. F., A manual of biochemistry, New York, 150 (1934).

56. Harrow, B., and Sherwin, C. P., A textbook of biochemistry, Philadelphia, 410 (1935).

57. Jorpes, E., *Biochem. J.*, **26**, 1507 (1932).

CHEMICAL STUDIES OF THE SUPRARENAL CORTEX

III. THE STRUCTURES OF COMPOUNDS A, B, AND H

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In preceding papers (10, 11) we have described the isolation and properties of five compounds and some of their degradation products. This paper presents a detailed study of Compounds A and B and a description of a new substance, Compound H, which is closely related to the other two.

Compounds A and B both have cortin-like physiologic activity and when tested by the Ingle (7) rat method Compound B was shown to be slightly more active than Compound A. Compound B was also tested on a suprarenalectomized dog which had been well standardized. At a level of 1.5 mg. daily the dog of 13.6 kilos weight was maintained in excellent condition but when the amount was reduced to 1.0 mg., the usual symptoms of suprarenal insufficiency soon appeared. These results are in accord with those described by Laqueur and coworkers (4) for the substance isolated by Reichstein and named "corticosterone." We have compared our Compound B with a sample of corticosterone kindly supplied by Professor Reichstein and have found them to be identical in every respect.

The formulas of Compounds A and B previously suggested must be revised on the basis of new determinations of their molecular weights. The molecular weight of Compound A was at first (10) determined by Rast's camphor method. It was noted that decomposition occurred slowly during the determination but the result was considered valid since a similar decomposition of Compound E did not affect appreciably the determination of the molecular weight, as was shown by other means. However, as soon as sufficient material was available, the molecular weight was determined by Menzies' (12) method, which did not involve any decomposition. The molecular weights of Compounds A and B were found to correspond to those of C_{21} instead of C_{26} compounds. The formula of Compound A is $C_{21}H_{28}O_4$ and that of Compound B, $C_{21}H_{30}O_4$. Compound H is $C_{21}H_{32}O_4$. Reichstein has not published a formula for corticosterone. His latest data (16) indicated a formula of $C_{19}H_{26}O_4$ or $C_{23}H_{32}O_5$ but since then (4) he has revised the values for the melting point and the specific rotation.

The relation of our Compounds A, B, and H which contain 4 atoms of oxygen to Compounds C, D, and E which contain 5 atoms of oxygen has not been established with certainty. However, the similarity in the physiologic activity of Compounds A, B, and E and the identity of certain details of their structure make it very probable that both series of compounds have the same structural skeleton. We have shown by physiologic means (11) (stimulation of comb growth in the capon) that Compound E has the steroid nucleus. It was also indicated that there was an oxygen atom at C_3 and that a side chain was attached at C_{17}. Reichstein (14, 15) has also shown by chemical as well as physiologic means that the compounds that contain 5 atoms of oxygen are steroid in nature. The precipitation by digitonin of Compound H and of Acid 1B which is derived from Compound A is very good evidence of their steroid nature. With the steroid nucleus as a basis sufficient evidence of structural details has been obtained to warrant the suggestion of probable structural formulas for Compounds A, B, and H and the substances derived from them. The suggested formulas are shown in Fig. 1.

Compound A (III) was obtained in yields of 0.7 to 1.0 gm. from 3000 pounds of fresh glands by crystallization of Fraction I from isopropyl alcohol. Fraction I is the fraction that remains in the benzene as the result of successive distributions between water and benzene. Compound A crystallized from the isopropyl alcohol as large prisms and was purified by repeated crystallizations from absolute alcohol. It did not give the fluorescence reaction with sulfuric acid described by Wintersteiner and Pfiffner (19).

Compound B (I) slowly separated as needles from the mother

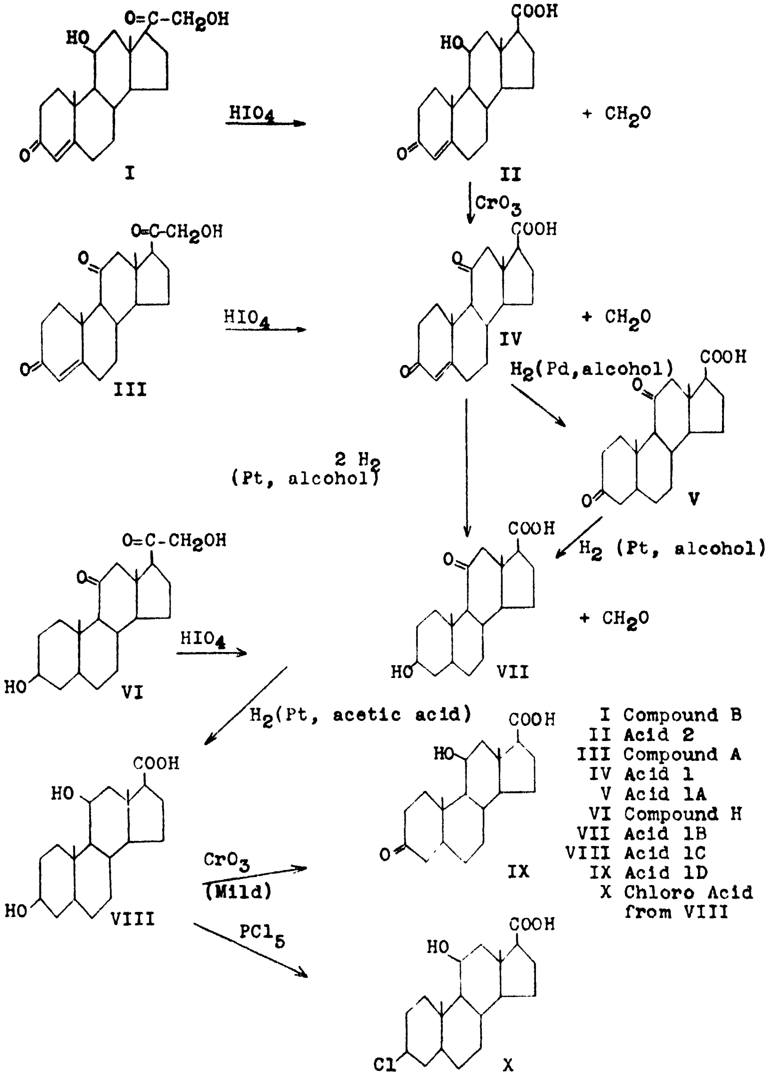

FIG. 1. Probable structural formulas

liquors of Compound A. Although the melting points of the two substances are identical (177–180°), the crystal forms, specific rotations (Compound A, +299°; Compound B, +258° for λ =

5461), and the melting point of a mixture (155–166°) showed they were different. For Compound B $[\alpha]_D^{25} = +222°$. Reichstein's (4) revised values for corticosterone are melting point 180–182° (corrected) and $[\alpha]_D^{15} = +223°$. The sample of Compound B that we described previously was evidently very impure but it was correctly termed the precursor of Acid 2. Compound B gave a strong fluorescence with sulfuric acid as was noted by Reichstein (16) for corticosterone. Examination in the Grignard machine revealed the presence of two hydroxyl and two carbonyl groups.

The presence of an α-ketol side chain in Compounds A and B was shown by oxidation with periodic acid. When the alcohol group is primary, this reagent oxidizes an α-ketol to formaldehyde and the corresponding acid with 1 less carbon atom; the consumption of periodic acid is equal to 1 atom equivalent of oxygen and the periodic acid is reduced to iodic acid (3). From Compound A, formaldehyde and Acid 1, $C_{20}H_{26}O_4$ (IV), and from Compound B, formaldehyde and Acid 2, $C_{20}H_{28}O_4$ (II), were obtained in equimolecular quantities with the use of periodic acid equal to 1 atom equivalent of oxygen. The formaldehyde was estimated and identified as the 2,4-dinitrophenylhydrazone and also as the dimedon derivative.

In view of the similar physiologic and chemical properties of Compounds A, B, and E, the α-ketol group of Compounds A and B is undoubtedly attached at the same position of the steroid nucleus as it is in Compound E; that is, most probably at C_{17}. Since Acid 1 has been found to have an α,β unsaturated ketone group (11), such a group must also be present in Compound A. Again on the basis of probability and analogy with Compound E this group has been assigned to the same position as in $\Delta^{4,5}$-cholestenone.

The determination of the nature of the 4th oxygen atom and its placement have been difficult but the discovery of the relation between Acid 1 and Acid 2 made clear the function of this oxygen atom in Compounds A and B. This relation was established by the conversion of Acid 2 to Acid 1 by oxidation with chromic acid. In addition to the carboxyl group Acid 2 has one hydroxyl and one ketone group while Acid 1 has two ketone groups, as determined with the Grignard reagent. It is evident then that Compound A has a ketone group in the same position that is occupied by a hydroxyl group in Compound B and that in all other respects they are identical.

The ketone group of Acid 1 that corresponds to the hydroxyl group of Acid 2 is exceedingly inert. It could not be made to react with hydroxylamine or 2,4-dinitrophenylhydrazine. It is stable to platinum and hydrogen in neutral alcohol and is reduced catalytically in acetic acid much more slowly than the other ketone group.

The study of the catalytic hydrogenation of Acid 1, which was carried out in detail, furnished sufficient additional information to permit the selection of a probable position for the 4th oxygen atom by comparison with other steroids of known structure. As would be expected by analogy with cholestenone, Acid 1 in alcohol readily absorbed 1 mole of hydrogen when shaken with palladium black. These conditions would be expected to produce a steric relation between the hydrogen atom at C_5 and the methyl group at C_{10} like that of coprostanone (5, 17). The consumption of hydrogen ceased when 1 mole had been absorbed. Substitution of Adams' platinum oxide catalyst for the palladium black caused a further rapid uptake of hydrogen which again virtually stopped after the absorption of a 2nd mole of hydrogen. Acid 1 was usually present as the sodium salt but the same results were obtained when the free acid was used. When the alcohol was replaced with acetic acid, a 3rd mole of hydrogen was consumed rather slowly. The acids obtained by this stepwise hydrogenation were isolated and designated Acids 1A, 1B, and 1C respectively.

Acid 1A (dihydro Acid 1) (V) readily gave a dinitrophenylhydrazone, showing the presence of the active ketone group. Its composition was established as $C_{20}H_{28}O_4$. Since the active ketone group was still present, only the double bond could have been reduced. The same acid was obtained by the oxidation with periodic acid of dihydro Compound A which was prepared by reduction of Compound A with palladium black and hydrogen.

Acid 1B (tetrahydro Acid 1) (VII) gave analytic values corresponding to the formula $C_{20}H_{30}O_4$. It did not react with carbonyl reagents. After prolonged heating with a solution of hydroxylamine the acid was recovered unchanged. It was attacked slowly by ozone, which led us to consider at one time the possibility of the presence of a double bond and a hydroxyl group instead of a carbonyl group. However, since the Grignard reagent showed the presence of 2 active hydrogen atoms and one carbonyl group that did not react completely, the inactive carbonyl group which is in Acid 1 was still intact. In 90 per cent alcohol Acid 1B gave a precipitate with digitonin.

Acid 1C (hexahydro Acid 1) (VIII), $C_{20}H_{32}O_4$, also was precipitated by digitonin in 90 per cent alcohol. The Grignard reagent showed 2.5 active hydrogen atoms. Phosphorus pentachloride or thionyl chloride in benzene or chloroform readily replaced one hydroxyl group with a chlorine atom but the other hydroxyl group was not attacked even on prolonged heating. The chloro acid (X) liberated 2 moles of methane from methyl magnesium iodide. It was concluded that the inactive ketone group of Acid 1 had been converted to a hydroxyl group which was quite inactive toward some reagents but would liberate methane from methyl magnesium iodide.

Acid 1D (IX) was obtained by the oxidation of Acid 1C (VIII) with a 0.3 N solution of chromic acid in aqueous acetone. Oxidation of both alcohol groups of Acid 1C would have yielded Acid 1A (V), while restoration of the inactive ketone group alone would have produced Acid 1B (VII). The product was neither Acid 1A nor Acid 1B and analysis of the acid and of its methyl ester showed that only 2 atoms of hydrogen had been lost by Acid 1C. Therefore the alcohol group at C_3 had been attacked, while the inert alcohol group had remained untouched.

Dihydro Acid 2 was prepared by the oxidation with periodic acid of 4,5-dihydro Compound B which was obtained by hydrogenation of Compound B in alcohol with palladium black as the catalyst. Dihydro Acid 2 should have the same structure as Acid 1D (IX) except for stereoisomerism. Although the melting points of the acids are not far apart, the specific rotations differ by 7°. The methyl esters have identical melting points but the melting point of a mixture of them was depressed 25°. The difference is attributed to epimerism of the alcohol group which in Acid 1D is resistant to mild treatment with chromic acid but in dihydro Acid 2 is readily oxidized with the formation of Acid 1A (V). This conclusion was confirmed by oxidation of Acid 1D to Acid 1A under less mild conditions.

The positions of the α-ketol side chain and the α,β unsaturated ketone group are reasonably certain although admittedly not proved. It remains then to find a position in which a ketone and a hydroxyl group could occur with the properties described. Although the correspondence in properties was not entirely satisfactory, C_{12} was first chosen as a possible situation (8). We have already stated in a brief note (9) that by synthesis of 3,12-diketoetiocholanic acid C_{12} has been excluded. Comparison of this acid with Acid 1A is now known to be inconclusive because of the uncertainty as to the configuration of C_5 in Acid 1A, as will be shown later. However, the exclusion of C_{12} appears to be valid in the light of a recent paper by Tschesche and Bohle[1] (18) in which a ketone group in sarmentogenone that corresponds in every way with the inactive ketone group of Acid 1 was described. Furthermore, the hydroxyl group formed by catalytic reduction of this ketone group in acetic acid solution cannot be replaced by heating with thionyl chloride in chloroform and cannot be acylated with benzoyl chloride in pyridine. This behavior is parallel with that of the hydroxyl group of Acid 1C that is formed by the reduction of the inactive carbonyl group of Acid 1 in acetic acid solution. The inactive ketone group of sarmentogenone was shown to be most probably located at C_{11}. On this basis Acid 1 would be 3,11-diketo-Δ-^{4,5}-etiocholenic acid. Work is under way to test these formulas.

Another substance related to Compounds A and B has been obtained from the mother liquors of Compound B and designated Compound H. Its formula is $C_{21}H_{32}O_4$. Periodic acid converted it to an acid, $C_{20}H_{30}O_4$, and formaldehyde. The acid proved to be identical with Acid 1B (VII). The structure of Compound H (VI) is therefore immediately apparent as tetrahydro Compound A. Furthermore, the catalytic reduction of Acid 1 produced the same configurations at C_3 and C_5 as those found in the natural product. Compound H was readily precipitated by digitonin.

The properties of the compounds discussed are summarized in Table I.

The stereochemistry of the reduction products of Acid 1 and of Compound H requires further consideration. It was anticipated from the work of Grasshof (5, 6) and Ruzicka (17) that with palladium as the catalyst in neutral solution the hydrogenation of the ethylenic bond of Acid 1 would result in a configuration at C_5

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[1] Grateful acknowledgment is given to Dr. J. J. Pfiffner, who drew our attention to this paper.

like that of coprostane and that reduction of the C_3 ketone group, again in neutral alcohol, would result in a configuration like that of epicoprosterol and the bile acids. This latter assumption is not in accord with the precipitation of Acid 1B and Acid 1C with digitonin. In these acids as well as in Compound H the hydroxyl group at C_3 must bear the same relation to the methyl group at C_{10} as does the hydroxyl group in cholesterol. There may have been some of the epimeric acid in the mother liquors after the separation of Acid 1B but since the yield of Acid 1B was over 85 per cent of the Acid 1 used, the reduction resulted chiefly in the one isomer. Since Acid 1B was found to be identical with the acid derived from Compound H, it is improbable that Acid 1B is a mixture or molecular compound of the two epimers.

TABLE I

Summary of Melting Points and Specific Rotations

Substance	Formula	M.p. (uncorrected)	Specific rotation $\lambda = 5461$	M.p., methyl ester (uncorrected)
		°C.	*degrees*	*°C.*
Compound A	$C_{21}H_{28}O_4$	177-180	+299	
Dihydro Compound A	$C_{21}H_{30}O_4$	174-176	+163	
Compound B	$C_{21}H_{30}O_4$	177-180	+258	
Dihydro Compound B	$C_{21}H_{32}O_4$	184-187	+157	
Hexahydro “ “	$C_{21}H_{36}O_4$	220-222	+39	
Compound H	$C_{21}H_{32}O_4$	172-176	+118	
Acid 1	$C_{20}H_{26}O_4$	267-269	+291	178-179
“ 1A	$C_{20}H_{28}O_4$	272-273	+114	200-201
“ 1B	$C_{20}H_{30}O_4$	272-274	+78	188-189
“ 1C	$C_{20}H_{32}O_4$	284-286	+71	
“ 1D	$C_{20}H_{30}O_4$	265-266	+93	170-171
“ 2	$C_{20}H_{28}O_4$	253-258	+218	
Dihydro Acid 2	$C_{20}H_{30}O_4$	265-270	+100	170-171

It is most likely that the hydrogenation of the ethylenic bond yielded as the main product the isomer with a configuration corresponding to that of cholestanone. In that case, the reduction of the ketone group in the neutral solution would be expected to give the result found; that is, the product would be of the dihydrocholesterol type but it is not apparent why the cholestane type should be obtained as the chief product in the first step of the hydrogenation. It may be noted, however, that when Butenandt and Mamoli (2) reduced 3-keto-$\Delta^{4,5}$-bisnorcholenic acid with hydrogen and palladium black in ether solution, over one-third of the product isolated was 3-ketobisnorallocholanic acid (cholestane type), whereas Grasshof (5) obtained only coprostanone when cholestenone was reduced under the same conditions. In any event the steric relations of Acid 1B and of Compound H at C_8 and C_5 are identical.

It is probable, then, that Compound H is of the dihydrocholesterol type. Reichstein (14, 15) has shown also that his Substance A which contains 5 atoms of oxygen has this type of structure.

It should be emphasized that Compounds A, B, and H contain 4 atoms of oxygen, while the other substances that have been described contain 5. Our results indicate that the repeated distributions between benzene and water effectively separate the two series of compounds. The series with 4 atoms of oxygen has been found exclusively in Fraction I which is the fraction more soluble in benzene, while the aqueous residues have yielded only the substances that contain 5 atoms of oxygen. It is not claimed that the separation is complete but it is sufficiently complete to permit the isolation of pure substances.

Hydrogenation of the double bond of Compounds A and B in alcohol with palladium black and hydrogen largely destroyed their activity, as tested by Ingle's method. The minimal quantity of Compounds A and B necessary in this test was 0.1 mg., while 5.0 mg. of the hydrogenated products were necessary to give a comparable test. Pfiffner, Wintersteiner, and Vars (13) showed on non-crystalline material that an α,β unsaturated ketone group was present in the fraction which possessed cortin activity. The reduction of the ethylenic bond in Compounds A and B demonstrates the essential nature of this grouping in regard to physiologic activity.

This paper has been confined to a discussion of the O_4 series of compounds. The O_5 series will be considered in a subsequent communication.

EXPERIMENTAL

The analytic procedures that were used have been discussed in the preceding papers. All samples for analysis were dried for at least 1 hour over anhydrous magnesium perchlorate under a pressure of less than 0.1 mm. and at a temperature of 110°. The melting points recorded in this paper are uncorrected. The methyl esters of the acids melted without decomposition. Compound B also melted without noticeable decomposition but all the other substances decomposed at their melting points. The higher melting points were somewhat variable on this account.

The data on active hydrogen atoms are recorded in terms of hydroxyl groups. It may be well to note that a carboxyl group accounts for 2 atoms of oxygen but gives evidence only of the active hydrogen atom.

Isolation of Compound A—Fraction I, as originally defined, was a combination of the residues that remained in the successive benzene solutions after distribution between benzene and water. However, since most of the material and most of Compound A are found in the first residue, this was the main source of Compound A. For its isolation the benzene was removed in a vacuum and the residue was dissolved in 4 volumes of isopropyl alcohol. One-fourth of the solvent was removed in a vacuum and the solution placed in the cold room. After 24 hours the crystals of Compound A were collected and washed with ice-cold isopropyl alcohol. As a typical example, 3000 pounds of fresh glands yielded 4.98 gm. of residue from which 711 mg. of crude Compound A were obtained. It was recrystallized to constant specific rotation from absolute alcohol. $[\alpha]^{25}_{5461} = +299° \pm 1°$ (0.8 per cent in alcohol). The melting point was 177-180°. A sharper melting point could not be obtained by further recrystallizations. At other times a larger yield of Compound A was obtained from the first residue. The subsequent residues yielded enough to raise the total weight of crude Compound A to 1.0 to 1.5 gm.

The molecular weight was determined by Menzies' method. The solvent was absolute alcohol. With a sample of 0.2089 gm., Δp was 33.0 mm. for a volume of 15.0 cc. and 25.5 mm. for a volume of 19.8 cc.

$C_{21}H_{28}O_4$.	Calculated.	C 73.21,	H 8.20,	mol. wt. 344.22
	Found.	" 73.45,	" 8.22,	" " 356
	"	" 73.36,	" 8.22,	" " 349

Acid 1. Preparation by Oxidation of Compound A with Periodic Acid—A number of oxidations were carried out under various

conditions. As an example, 688 mg. (0.002 mole) of Compound A in 160 cc. of alcohol were treated with 95 cc. of 0.025 M periodic acid, 3 cc. of 5 N sulfuric acid, and 60 cc. of water. After 15 hours the solution was concentrated to a small volume in a vacuum, the distillate being received in a solution of dimedon (dimethyldihydroresorcinol). Water was added and the distillation continued until the volume was again small, when another portion of water was added. Repetition of this process several times resulted in the formation of 580 mg. of formal-dimedon which melted at 192°. A mixture of this substance with an authentic specimen of formal-dimedon also melted at 192°. If each molecule of Compound A yields 1 molecule of formaldehyde, 584 mg. of the dimedon derivative would be expected.

The periodic acid used would have liberated iodine equivalent to 190.0 cc. of 0.1 N thiosulfate. After the oxidation, 150.4 cc. of 0.1 N iodine were liberated. The periodic acid reduced was equivalent to 39.6 cc. of 0.1 N thiosulfate or 0.00198 mole of oxygen. Therefore Compound A was oxidized with 1 atom equivalent of oxygen and 1 molecule of formaldehyde was produced. In another experiment the formaldehyde was collected in a solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid (Brady's reagent). The derivative melted at 164.5° and when mixed with a specimen of formaldehyde 2,4-dinitrophenylhydrazone still melted at 164.5°.

A large proportion of the acid formed by the oxidation crystallized as the alcohol was removed. It was dissolved in ether and the solution was also extracted with ether. The combined ether solutions were extracted with a solution of sodium bicarbonate which in turn was washed once with ether. Acidification of the bicarbonate solution yielded 547 mg. (83 per cent) of Acid 1, which was purified by crystallization from absolute alcohol. Its melting point, 267–269°, was sharper than that previously recorded. $[\alpha]_{5461}^{25} = +291°$ (0.3 per cent in alcohol). The molecular weight was checked by titration; 18.4 mg. required 4.73 cc. of 0.0117 N sodium hydroxide.

$C_{20}H_{26}O_4$. Calculated. C 72.68, H 7.93, mol. wt. 330
Found. " 72.85, " 8.05, " " 332

The distribution of the oxygen atoms of Acid 1 as determined with the Grignard reagent has been given previously (10). The determination was repeated with the new preparation and the presence of one active hydrogen and two carbonyl groups confirmed.

$C_{20}H_{26}O_4$. Calculated, 1OH 5 15, 2CO 17 0; found, OH 6.46, CO 19.3

The Acid 1 prepared as just described was more easily purified than the preparation previously made with chromic acid. The latter gave a strong yellow color with tetranitromethane but the preparations made with periodic acid do not give a color.

The methyl ester of Acid 1 was prepared with diazomethane. It melted at 178–179°.

$C_{21}H_{28}O_4$. Calculated, C 73 21, H 8.20; found, C 73.05, H 8.38

The oxime of 10 mg. of Acid 1 was prepared with 10 mg. of hydroxylamine hydrochloride and 20 mg. of sodium acetate in 2 cc. of alcohol. After 2 hours of refluxing 2 cc. of water were added and the solution was evaporated to 0.5 cc. The product was recrystallized from dilute alcohol and had the composition of a monooxime. It melted at 258–260°.

$C_{20}H_{27}O_4N$. Calculated, C 69 52, H 7 89; found, C 69 49, H 8 09

Isolation of Compound B—The isopropyl alcohol solution from which Compound A (711 mg.) had been removed was evaporated under reduced pressure to about one-half the original volume. After 24 hours in the cold room a second crop of 715 mg. of crystals was filtered out and washed with ice-cold isopropyl alcohol. Two recrystallizations from the same solvent yielded 133 mg. of material with a melting point, 177–180°, that could not be distinguished from that of Compound A. The specific rotation, however, served to identify this material as another substance. $[\alpha]^{25}_{5461} = +258° \pm 3°$; $[\alpha]^{25}_{D} = +222°$ (0.6 per cent in alcohol). Analysis and determination of the molecular weight indicated a formula of $C_{21}H_{30}O_4$. Δp = 21.0 mm. and 19.5 mm. at 24.0 cc. and 26.1 cc. respectively.

$C_{21}H_{30}O_4$	Calculated.	C 72.78,	H 8.73,	mol. wt. 346.24
	Found.	" 72 85,	" 8.82,	" " 368
	"	" 72 75,	" 8.87,	" " 365

By working up the mother liquors a total of 501 mg. of Compound B was obtained. The subsequent benzene residues produced enough to raise the total yield of crude Compound B to 1.10 gm. from which 775 mg. of the pure substance were obtained.

The physical constants and analytic values are different from those originally given for Compound B. Reichstein (16) also appears to have had some difficulty in obtaining this substance in a state of purity. His first analytic figures for his Substance H (C 71.22, H 8.34) were in agreement with our preliminary values (C 71.55, H 8.76). Later analyses by Reichstein are not available but the melting point (181–182°) and the specific rotation ($[\alpha]_D^{15}$ = +223°) published by Reichstein and coworkers (4) for their Substance H, now called corticosterone, agree with our present values. Admixture of corticosterone supplied by Professor Reichstein did not depress the melting point of our Compound B. It also gave the fluorescence reaction with sulfuric acid, as was mentioned by Reichstein.

The analysis with the Grignard reagent indicated the presence of two hydroxyl and two carbonyl groups, although one of the latter did not react completely.

$C_{21}H_{30}O_4$. Calculated, 2OH 9.84, 2CO 16.2; found, OH 10.3, CO 13.2

Acid 2. Preparation by Oxidation of Compound B with Periodic Acid—The procedure was essentially the same as for Compound A. For 50 mg. (0.000144 mole) of Compound B, periodic acid equal to 0.000144 atom equivalent of oxygen was used. A quantitative recovery of formaldehyde was not attempted. It was isolated from the first distillate in a yield of 49 per cent as the 2,4-dinitrophenylhydrazone (14 mg.) which melted at 164.5°. During the removal of the alcohol fine needles separated, which weighed 34 mg. After solution in very dilute sodium hydroxide and precipitation with acid the substance melted at 253–258° with decomposition. $[\alpha]_{5461}^{25}$ = +218° ± 2° (0.34 per cent in alcohol). Analysis indicated the formula $C_{20}H_{28}O_4$. For the neutralization of 13.9 mg., 3.55 cc. of 0.0117 N sodium hydroxide were required.

$C_{20}H_{28}O_4$.	Calculated.	C 72 24,	H 8.49,	mol. wt. 332
	Found.	" 72 15,	" 8.46,	" " 334

The preparation of an acid with the same composition and properties by oxidation of amorphous material with chromic acid was described in our first paper and labeled Acid 2. Compound B was correctly suggested as its precursor.

The presence of 2 active hydrogen atoms and one carbonyl group was shown with the Grignard reagent.

$C_{20}H_{28}O_4$. Calculated, 2OH 10.2, 1CO 8.44; found, OH 10.2, CO 10.1

Oxidation of Acid 2 to Acid 1—To 37 mg. of Acid 2 in 5 cc. of acetone were added 2 cc. of 2 N potassium dichromate solution and 6 cc. of N sulfuric acid. The mixture stood overnight. On removal of the acetone in a vacuum 28 mg. of crystals separated. These were recrystallized from absolute alcohol. The melting point (258–260°) and specific rotation (+287° ± 3°) were those of Acid 1. A mixture with Acid 2 melted at 218–228°. Analysis and comparison of the methyl esters confirmed the identity of oxidized Acid 2 and Acid 1.

$C_{20}H_{26}O_4$. Calculated, C 72.68, H 7.93; found, C 72.50, H 8.03

The methyl ester of the acid obtained by the oxidation of Acid 2 was prepared with diazomethane. It melted at 178–179°. When it was mixed with a sample of the methyl ester of Acid 1 the melting point was 178–179°.

Hydrogenation of Compound A—The sample (97.3 mg. = 0.000283 mole) was dissolved in 10 cc. of absolute alcohol and the solution was shaken with hydrogen and 28 mg. of palladium black until absorption of hydrogen ceased. The hydrogen consumed was 6.7 cc.; the amount calculated for 1 mole was 6.3 cc. After removal of the catalyst and distillation of the alcohol under reduced pressure the residue was taken up in acetone. Addition of one-third the volume of water caused the separation of 79 mg. of dihydro Compound A which melted at 174–176°. $[\alpha]_{5461}^{25} = +163° \pm 4°$ (0.40 per cent in alcohol).

$C_{21}H_{30}O_4$. Calculated, C 72.78, H 8.73; found, C 72.55, H 8.95

This preparation was tested for physiologic activity by Ingle's method (7) as described for dihydro Compound B. It was perhaps slightly less active than dihydro Compound B.

Acid 1A. Preparation by Oxidation of Dihydro Compound A—The oxidation with periodic acid was carried out in a very dilute aqueous solution. A little alcohol was used to dissolve 43 mg. of the substance. After addition of 175 cc. of water the alcohol was removed under reduced pressure. The other additions were 18 cc. of 0.03 M periodic acid and 1 cc. of 5 N sulfuric acid. The next morning the solution was evaporated in a vacuum to a volume of 30 cc. The acid that separated weighed 21 mg. (80 per cent of 1 mole). The acid melted at 272–273° and the methyl ester melted at 200–201°. These are the melting points of Acid 1A and its methyl ester. The appropriate mixtures with authentic specimens possessed the same melting points. Acid 1A is a dihydro Acid 1 and is described later. The experiment served to prove that hydrogen added to Compound A and Acid 1 in the same way.

Hydrogenation of Compound B—The catalyst (40 mg. of palladium black) was first shaken with 10 cc. of absolute alcohol and hydrogen until equilibrium was reached. Then 98 mg. (0.000283 mole) of Compound B were added. The absorption of hydrogen became very slow when almost 1 mole of hydrogen had been consumed. The hydrogen used was 5.6 cc.; calculated for 1 mole 6.3 cc. The catalyst and alcohol were removed as usual and the residue was taken up in acetone. The dihydro Compound B (60 mg.) crystallized on addition of an equal volume of water. It melted at 184–187°. $[\alpha]_{5461}^{25} = +157° \pm 3°$ (0.44 per cent in alcohol).

$C_{21}H_{32}O_4$. Calculated, C 72.36, H 9.26; found, C 72.58, H 9.54

This material was used in the Ingle rat test. 5 mg. in maize oil were required every 12 hours to maintain the animals in such a condition that they were able to work as well as the animals that received 0.1 mg. of Compound B dissolved in oil.

The reduction of 45 mg. of dihydro Compound B in absolute alcohol was continued with 40 mg. of platinum oxide catalyst until uptake of hydrogen ceased. 2 moles were consumed (used 6.3 cc., calculated for 2 moles 5.8 cc.). The product, after removal of catalyst and alcohol, crystallized on dilution of its acetone solution with an equal volume of 50 per cent alcohol. The hexahydro Compound B (32 mg.) melted at 220–222.5°. $[\alpha]_{5461}^{25} = 39° \pm 4°$ (0.28 per cent in alcohol).

$C_{21}H_{36}O_4$. Calculated, C 71.59, H 10.23; found, C 71.55, H 10.20

Dihydro Acid 2. Preparation by Oxidation of Dihydro Compound B with Periodic Acid—The procedure was similar to that used for dihydro Compound A. From 67.6 mg. (0.000194 mole) of substance were obtained 53.5 mg. of acid and 24.5 mg. of formaldehyde 2,4-dinitrophenylhydrazone with a consumption of periodic acid equal to 0.000196 atom equivalent of oxygen. The acid was dissolved in dilute sodium hydroxide and reprecipitated with hydrochloric acid. The crystalline acid was recrystallized from an acetone-water solution. This dihydro Acid 2 melted at 265–270°. $[\alpha]_{5461}^{25} = +100° \pm 2°$ (0.35 per cent in alcohol).

$C_{20}H_{30}O_4$. Calculated. C 71.80, H 9.05; found, C 72.02, H 9.20

The methyl ester was prepared with diazomethane. It melted at 170–171°. A mixture with the methyl ester of Acid 1D (melting point 170–171°) which is described later melted at 140–145°.

Acid 1A. Preparation by Oxidation of Dihydro Acid 2—When dihydro Acid 2 was oxidized with chromic acid under the same conditions used for the oxidation of Acid 2 to Acid 1, the product was Acid 1A (dihydro Acid 1). After crystallization from absolute alcohol the melting point was 273–275°. A mixture with a known sample of Acid 1A melted at the same temperature, while a mixture with dihydro Acid 2 melted at 260–268°.

Hydrogenation of Acid 1

A. Preparation of Acid 1A—The reduction of Acid 1 was carried out in steps. The first step involved the reduction of the α,β-ethylenic bond only. This was shown by the fact that the product, Acid 1A, readily yielded a yellow 2,4-dinitrophenylhydrazone. The active ketone group was still present but no longer conjugated with a double bond. α,β unsaturated ketones give 2,4-dinitrophenylhydrazones that are red or red-orange in color.

Palladium black was used as the catalyst for the first stage of the hydrogenation, since Grasshof (5, 6) has shown that under the influence of this catalyst only the double bond of cholestenone and similar compounds is reduced. Acid 1 (250 mg.) was dissolved in 15 cc. of absolute alcohol and shaken with hydrogen and 40 mg. of palladium black. The uptake of hydrogen ceased when 1 mole had been used (calculated 17.0 cc.; found 17.3 cc.). The catalyst and alcohol were removed and the residue dissolved in acetone. When the solution was evaporated to a volume of 3 to 5 cc., the acid separated. It was washed with dry acetone and then melted at 272–273°. $[\alpha]^{25}_{5461}$ = +114° ± 2° (1.0 per cent in alcohol).

$C_{20}H_{28}O_4$. Calculated, C 72.25, H 8.49; found, C 71.90, H 8.72

Acid 1A (20 mg.) was treated with an excess of hydroxylamine in the usual way. Refluxing in alcohol (3 cc.) was continued for 3 hours. The alcohol was largely removed and 10 cc. of water were added. The precipitate was dissolved in sodium carbonate solution and reprecipitated with acetic acid. It separated from absolute alcohol as small, discrete particles but without definite crystalline form. The analysis indicated that it was the monoöxime of Acid 1A.

$C_{20}H_{29}O_4N$. Calculated, C 69.12, H 8 42; found, C 69.17, H 8.77

B. Preparation of Acid 1B—For this preparation 163 mg. (0.000494 mole) of Acid 1 were dissolved in 5 cc. of alcohol and 4.98 cc. of 0.1 N sodium hydroxide. The solution was shaken with hydrogen and 13 mg. of platinum oxide catalyst (Adams and Shriner) until there was no further absorption of hydrogen. 2 moles were used (calculated 22.1 cc., found 21.3 cc.). The solution was filtered and the alcohol distilled. The residue was dissolved in 50 cc. of water to which were added 50 cc. of a saturated solution of barium chloride. This solution was evaporated until crystals of barium chloride began to separate. It was then cooled and filtered. The barium salts were dissolved in water and the Acid 1B precipitated with hydrochloric acid. For purification an acetone solution was diluted with water and warmed on the steam bath until the acetone was gone. After cooling the acid was collected, washed with water, and dried. The yield was 142 mg. of Acid 1B which melted at 272–274°. $[\alpha]^{25}_{5461}$ = +78° ± 2° (0.39 per cent in alcohol). It was readily precipitated from a solution in 90 per cent alcohol by digitonin.

$C_{20}H_{30}O_4$. Calculated. C 71.80, H 9 05, 2OH 10 2, 1CO 8 39
Found. " 71.86, " 8.97, OH 10.8, CO 5.62

The methyl ester of Acid 1B was prepared by treatment with diazomethane in ether solution. It was crystallized from methanol and melted at 188–189°.

$C_{21}H_{32}O_4$. Calculated, C 72 36, H 9.26; found, C 72.01, H 9.23

Acid 1B (22 mg.) was refluxed for 3 hours in an alcoholic solution containing 10 times the calculated amount (for 2CO) of hydroxylamine acetate. The material recovered (19 mg.) melted at 269–273° and did not contain nitrogen. The failure of Acid 1B to combine with hydroxylamine is in accord with the formation of a monooxime by Acid 1. The ketone group did not react completely in the test with the Grignard reagent.

C. Preparation of Acid 1C—The platinum oxide catalyst (46 mg.) was reduced in 10 cc. of glacial acetic acid. To this were added 93 mg. of Acid 1B. The reduction proceeded slowly and stopped when 1 mole of hydrogen had been absorbed (calculated 6.2 cc., found 6.4 cc.). After filtration the acetic acid was removed under reduced pressure. The residue was converted into a crystalline mass by repeated distillations under reduced pressure with benzene. It was then slightly soluble in cold, dry acetone from which it was recrystallized. The melting point was 284–286°; $[\alpha]^{25}_{5461} = +71° \pm 3°$ (0.19 per cent in alcohol). Acid 1C was also precipitated by digitonin.

$C_{20}H_{32}O_4$. Calculated. C 71.38, H 9.60, 3OH 15.2
Found. " 71.56, " 9.68, OH 12.7

Acid 1C liberated only 2.5 moles of methane from the Grignard reagent. The deficiency probably resulted from the unfavorable nature of the precipitate that was formed on addition of the Grignard reagent. When one hydroxyl group was replaced by chlorine, then the 2 remaining active hydrogen atoms liberated their full equivalent of methane.

Acid 1A. Preparation by Oxidation of Acid 1B—A mixture of 10.5 cc. of 2 N potassium dichromate solution, 21 cc. of N sulfuric acid, and 10 cc. of water was added to 240 mg. of Acid 1B dissolved in 21 cc. of acetone. After 20 hours at room temperature the acetone was removed under reduced pressure and the aqueous residue saturated with ammonium sulfate. The solution was extracted with ether which was then dried with sodium sulfate and distilled. The residue was crystallized from acetone and melted at 272–274°. When mixed with Acid 1A there was no depression of the melting point. $[\alpha]^{25}_{5461} = +112° \pm 2°$. The yield was 198 mg. The reduction of Acid 1 with palladium and with platinum catalysts was thus shown to produce the same configuration at C_5.

Acid 1D. Preparation by Oxidation of Acid 1C—A solution of 16 mg. of Acid 1C in 5 cc. of acetone was treated with 2.5 cc. of 2 N potassium dichromate solution and 5 cc. of N sulfuric acid. After 2 hours the acetone was removed under reduced pressure. The product was filtered out of the aqueous residue, washed with water, and recrystallized from acetone-water. The yield was 11 mg. of Acid 1D which melted at 265–266°. $[\alpha]_{5461}^{25} = +93° \pm 2°$ (0.1 per cent in alcohol).

$C_{20}H_{30}O_4$. Calculated, C 71.80, H 9.05; found, C 71.83, H 8.95

The methyl ester of Acid 1D was prepared with diazomethane and crystallized from acetone-water. It melted at 170–171°. This is also the melting point of the methyl ester of the dihydro Acid 2 described under the oxidation of dihydro Compound B but it was noted there that the two esters were different.

$C_{21}H_{32}O_4$. Calculated, C 72 36, H 9.26; found, C 72.45, H 9.27

Oxidation of Acid 1C to Acid 1B—Although Acid 1D contained the inert alcohol group which had survived the mild treatment with chromic acid, it was shown that less mild conditions resulted in the oxidation of this group. When Acid 1C was heated at 100° with acetic anhydride, the monoacetate was formed, as is evident from the results to follow. The acetate (40 mg.) was treated with chromic acid (133 mg.) in glacial acetic acid (2 cc.) for 3 hours and the product was isolated in the usual way. The melting point was 210–213°. That is also the melting point of the acetate prepared by the action of acetic anhydride on Acid 1B. A mixture of the two specimens melted at the same temperature.

$C_{22}H_{32}O_5$. Calculated, C 69.90, H 8.80; found, C 70.17, H 8.70

After hydrolysis the acid melted at 272–273° and a mixture with an authentic specimen of Acid 1B melted at 272–273°.

Action of Phosphorus Pentachloride on Acid 1C—A suspension of 100 mg. of phosphorus pentachloride in 3 cc. of dry chloroform was added to a solution of 40 mg. of Acid 1C in 5 cc. of dry chloroform. The mixture was stirred at room temperature until all had dissolved. It was then poured on ice. The chloroform was separated, washed with water, and distilled. The residue was crystallized from alcohol. The first portion that separated melted at

200–203° while the second crop melted at 214–217°. The first fraction had a chlorine content of 11.5 per cent. The composition of the second fraction was somewhat closer to that calculated for the acid containing 1 atom of chlorine.

$C_{20}H_{31}O_3Cl$. Calculated, Cl 9 99, 2OH 9.58; found, Cl 10.37, OH 9.92

The high chlorine content suggested that a 2nd atom of oxygen had been replaced with chlorine to some extent. Accordingly, a larger proportion of phosphorus pentachloride (1 gm.) was used with the methyl ester of Acid 1C (120 mg.). The procedure was essentially as before. The crude product was induced to crystallize by repeated distillations under reduced pressure of the acetone from an acetone-water solution. It was finally recrystallized from an acetone-water solution and then melted at 128–130°. Alkaline hydrolysis yielded the same monochloro acid described in the preceding paragraph. The melting point was 216°.

The methyl ester of the monochloro acid (96 mg.) was refluxed in thionyl chloride (3 cc.) for 3 hours. After evaporation of the thionyl chloride the product was crystallized from an acetone-water solution. The melting point was 128–130° and was not depressed by admixture of the starting material.

Isolation of Compound H—Several filtrates from the separation of Compound B were combined and the solvent was removed. The residue, 9.7 gm., was dissolved in 50 cc. of alcohol. To this were added 3 liters of water that contained 2 gm. of sodium chloride. The resulting solution was evaporated under reduced pressure. At a volume of 2.5 liters it was filtered from a dark colored gum. The clear solution was further concentrated to 1 liter and again filtered from 2 gm. of gum. This latter portion of gum was fractioned in a similar way from 2 liters of water. When the volume of this solution reached 1 liter, 294 mg. of gum were removed. Evaporation was continued until the volume reached 300 cc. The solution was then allowed to stand. At the end of 2 days 502 mg. of crystalline material with $[\alpha] = +172°$ had separated. This was dissolved in 5 cc. of alcohol and water was added until crystals began to separate. The first crop of 200 mg. was recrystallized twice in the same way and yielded 126 mg. with $[\alpha]^{25}_{5461} = +118°$ (0.62 per cent in alcohol). A third treatment did not change the specific rotation. The melting point was 172–176°.

In 90 per cent alcohol solution digitonin gave a copious precipitate with Compound H.

$C_{21}H_{32}O_4$. Calculated, C 72.36, H 9.26; found, C 72.52, H 9.27

Acid 1B. Preparation by Oxidation of Compound H with Periodic Acid—The oxidation with periodic acid was carried out as before with 88 mg. (0.00023 mole) of Compound H and 21 cc. of 0.03 M periodic acid. The periodic acid consumed was equal to 0.000243 atom equivalent of oxygen. Formaldehyde was identified as the 2,4-dinitrophenylhydrazone but only that quantity (40 per cent of 1 mole) that appeared in the distillate from the concentration of the original solution was recovered. A quantitative recovery was not attempted. The acid that separated on concentration of the solution was dissolved in water as the sodium salt and the solution was washed with ether. Acidification with dilute sulfuric acid yielded 69.5 mg. of acid which was recrystallized from an acetone-water solution. It melted at 270–273°, and $[\alpha]^{25}_{5461} = +81°$ (0.20 per cent in alcohol). This acid was also precipitated by digitonin from its solution in 90 per cent alcohol.

$C_{20}H_{30}O_4$. Calculated, C 71.80, H 9.05; found C 71.84, H 9.31

This acid did not combine with 2,4-dinitrophenylhydrazine. The formula indicated the presence of either a ketone group or ethylenic bond. The presence of the inactive ketone group of Acid 1 seemed the most probable, since the absence of a ketone group at C_3 was indicated by the failure to combine with dinitrophenylhydrazine. The assumed structure was shown to be correct by comparison with Acid 1B. Except for possible stereoisomerism the two acids should be identical. The melting points and specific rotations were in good agreement and a mixture of the new acid with Acid 1B melted at 269–272°. The sample of Acid 1B used melted at 270–272°. For further comparison of melting points the methyl esters were chosen because they do not decompose on melting. The esters were prepared with diazomethane, recrystallized from 50 per cent alcohol, and dried at 105°. The ester of the acid derived from Compound H melted at 186–188°; the ester of Acid 1B at 186–188° and a mixture of the two at 186–188°.

As a further check on the identity of the acid obtained from Compound H it was oxidized with chromic acid. For this 18 mg.

of the acid were oxidized in 6 cc. of acetone, 1 cc. of 2 N potassium dichromate, and 0.4 cc. of 5 N sulfuric acid. After 3 hours at room temperature the acetone was removed and 20 cc. of water were added. The acid was collected, washed with water, and recrystallized from aqueous acetone. The weight was 12 mg. $[\alpha]^{25}_{5461} = +113°$ (0.13 per cent in alcohol); for Acid 1A $[\alpha]^{25}_{5461} = +114° \pm 2°$. The melting point was 269–271° and a mixture with Acid 1A melted at 269–271°. The oxidized acid gave a yellow 2,4-dinitrophenylhydrazone as would be expected of Acid 1A.

The identification of the acid derived from Compound H and the data from the oxidation with periodic acid complete the identification of Compound H as a tetrahydro Compound A with the α,β unsaturated ketone group of Compound A reduced to the saturated secondary alcohol.

SUMMARY

Compounds A and B, previously described, and Compound H, recently isolated, have been studied in detail. They are C_{21} steroids with α-ketol side chains assigned to C_{17} as in other steroids. Compounds A and B have an α,β unsaturated ketone group which is assigned to the same position as in progesterone. In Compound H the ethylenic bond is absent and an alcohol group takes the place of the ketone. In Compounds A and H there is an inert ketone group which is assigned to C_{11}. An alcohol group occupies this position in Compound B. The inert ketone group has been assigned to C_{11} because of the close correspondence of its properties with those of the inert ketone group at C_{11} in sarmentogenone.

Compounds A and B have cortin-like physiologic activity and Compound B is identical with the corticosterone of Reichstein (4). The α,β unsaturated ketone group has been related to physiologic activity.

BIBLIOGRAPHY

1. Adams, R., and Shriner, R. L., *J. Am. Chem. Soc.*, **45**, 2171 (1923).
2. Butenandt, A., and Mamoli, L., *Ber. chem. Ges.*, **68**, 1854 (1935).
3. Clutterbuck, P. W., and Reuter, F., *J. Chem. Soc.*, **2**, 1467 (1935).
4. de Fremery, P., Laqueur, E., Reichstein, T., Spanhoff, R. W., and Uyldert, I. E., *Nature*, **139**, 26 (1937).
5. Grasshof, H., *Z. physiol. Chem.*, **223**, 249 (1934).
6. Grasshof, H., *Z. physiol. Chem.*, **225**, 197 (1934).

7. Ingle, D. J., *Am. J. Physiol.*, **116**, 622 (1936).
8. Kendall, E. C., Mason, H. L., Hoehn, W. M., and McKenzie, B. F., *Proc. Staff Meetings Mayo Clin.*, **12**, 136 (1937).
9. Kendall, E. C., Mason, H. L., Hoehn, W. M., and McKenzie, B. F., *Proc. Staff Meetings Mayo Clin.*, **12**, 270 (1937).
10. Mason, H. L., Myers, C. S., and Kendall, E. C., *J. Biol. Chem.*, **114**, 613 (1936).
11. Mason, H. L., Myers, C. S., and Kendall, E. C., *J. Biol. Chem.*, **116**, 267 (1936).
12. Menzies, A. W. C., *J. Am. Chem. Soc.*, **32**, 1615 (1910).
13. Pfiffner, J. J., Wintersteiner, O., and Vars, H. M., *J. Biol. Chem.*, **111**, 585 (1935).
14. Reichstein, T., *Helv. chim. acta*, **19**, 402 (1936).
15. Reichstein, T., *Helv. chim. acta*, **19**, 979 (1936).
16. Reichstein, T., *Helv. chim. acta*, **19**, 1107 (1936).
17. Ruzicka, L., Brüngger, H., Eichenberger, E., and Meyr, J., *Helv. chim. acta*, **17**, 1407 (1934).
18. Tschesche, R., and Bohle, K., *Ber. chem. Ges.*, **69**, 2497 (1936).
19. Wintersteiner, O., and Pfiffner, J. J., *J. Biol. Chem.*, **116**, 291 (1936).

THE DETERMINATION OF THIOL AND DISULFIDE COMPOUNDS, WITH SPECIAL REFERENCE TO CYSTEINE AND CYSTINE

VIII. MOLECULAR RATIO BETWEEN A-PHOSPHO-18-TUNGSTIC ACID AND CYSTEINE IN THEIR COLOR REACTION

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It has been reported by the author (1–4) that the blue color produced from A-phospho-18-tungstic acid with sulfhydryl compounds and some other reducing substances is strictly proportional to the concentration of the reductant and is stable at pH 5 for more than 6 hours at room temperature. It was suggested that the reaction can be represented by the equation

$$2R{-}SH + (H_2O)_3P_2O_5(WO_3)_{18} = R{-}S{-}S{-}R + H_2O + (H_2O)_3P_2O_5(WO_3)_{17}WO_2$$
$$\text{or } (H_2O)_3P_2O_5(WO_3)_{16}W_2O_5$$

It is now found, however, that the reaction is not completely represented by this equation and appears to consist of two steps, the first of which is rapid and the second slow.

According to the experiments of Wu (5), 1 molecule of A-phospho-18-tungstic acid loses 1 atom of oxygen on treatment with 2 molecules of ferrous sulfate or 1 of uric acid when the reaction is carried out at high alkalinity. However, it has been shown by Lugg (6) and by the present author (1) that the color developed at pH values above 8 is greater than that developed below this level, and that below pH 4.7 the color development is slow and the maximum intensity reached is less than that obtained over the range pH 4.7 to 7.5. Moreover, the color intensity remains constant only in this latter region and in presence of an excess of the complex acid. It therefore seems probable that the chemical relations between cysteine and the complex acid in this range are different from those that obtain above and below it. The experimental results here reported confirm this view.

EXPERIMENTAL

Ammonium A-Phospho-18-Tungstate—To 2 liters of the reagent solution, prepared as reported in Paper I (1), ammonium chloride (about 110 gm.) was added until no further precipitation occurred. The light blue crystalline product (about 170 gm.) was filtered off, dissolved in 250 cc. of water, decolorized by a drop of bromine, and reprecipitated by the addition of ammonium chloride. The precipitate was dissolved in 100 cc. of hot water, and the large crystals which separated on cooling were recrystallized in the same manner.

The yellow crystals so obtained corresponded in form and composition to those described by Wu (5).

Analysis—$(NH_3)_6P_2O_5(WO_3)_{18}(H_2O)_{19}$

Calculated. NH_3 2.15, WO_3 87.68, P_2O_5 2.98

Found. " 2.17, " 87.71, " 2.38

The 0.01 M solution used for color production was made by dissolving 11.908 gm. in 250 cc. of water.

A-Phospho-18-Tungstic Acid—The reagent solution (1) was evaporated at 70° until large crystals separated on cooling. After recrystallization they showed the composition $P_2O_5(WO_3)_{18}(H_2O)_{26}$.

Calculated. P_2O_5 2.97, WO_3 87.25, H_2O 9.79

Found. " 2.86, " 87.21, " 9.90

Wu assigned to the free acid the formula $P_2O_5(WO_3)_{18}(H_2O)_{38}$. The discrepancy is probably due to differences in the manner of drying.

The 0.01 M solution of the acid was made by dissolving 11.966 gm. of the crystals in 250 cc. of water.

Color Production—Definite quantities of solutions of the ammonium salt or of the free acid were added to mixtures of 10 cc. of 2 M sodium acetate and 3 cc. of 2 M acetic acid in a 50 cc. flask. After the addition of suitable amounts of water and shaking, varying quantities of 0.01 M cysteine (99.5 per cent pure) in 0.2 M hydrochloric acid were added and the mixture diluted to 50 cc.

Whenever the cysteine solution exceeded 2 cc., equivalent amounts of sodium hydroxide solution were added.

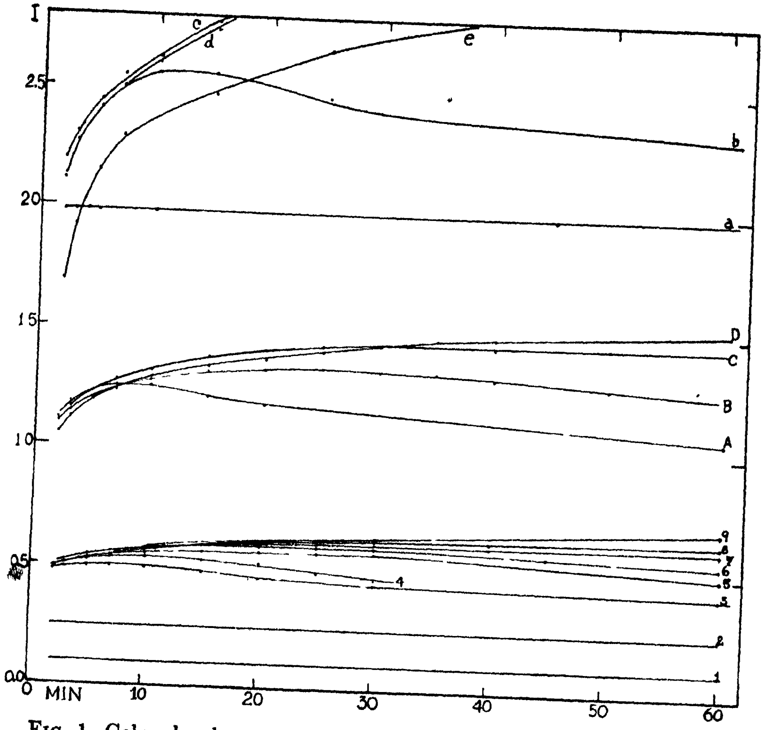

Fig. 1. Color development by ammonium A-phospho-18-tungstate and cysteine (25° ± 3.0°). Composition of the color solutions: Curves 1 to 9, 2.0×10^{-4} M ammonium A-phospho-18-tungstate and the following concentrations of cysteine ($\times 10^{-4}$ M). Curve 1, 0.4; Curve 2, 1.0; Curve 3, 2.0; Curve 4, 2.2; Curve 5, 2.4; Curve 6, 2.6; Curve 7, 3.0; Curve 8, 4.0; Curve 9, 10.0. Curves A to D, 5.0×10^{-4} M ammonium A-phospho-18-tungstate and cysteine ($\times 10^{-4}$ M). Curve A, 5.0; Curve B, 5.4; Curve C, 6.0; Curve D, 10.0. Curves *a* to *e*, 10.0×10^{-4} M ammonium A-phospho-18-tungstate and cysteine ($\times 10^{-4}$ M). Curve *a*, 8.0; Curve *b*, 10.0; Curve *c*, 11.0; Curve *d*, 12.0; Curve *e*, 20.0. Medium, 0.4 M sodium acetate and 0.12 M acetic acid. Color standard, 4×10^{-4} M cysteine with 4 cc. per 50 cc. of the reagent solution. *I* represents color intensity.

The color was read after 2 minutes and subsequently at definite intervals of time. The solution containing 2 cc. of 0.01 M cysteine and 4 cc. of the reagent solution was taken as the color standard as in previous experiments. The results are shown in Figs. 1, 2, and 3.

The results presented in Fig. 1 indicate that when the molarity of cysteine is lower than that of the ammonium salt, maximum color intensities are reached before 2 minutes and thereafter remain constant (probably for more than 6 hours). When the molarity of cysteine is equal to, or slightly greater than, that of the reagent, the color gradually fades after having reached the expected maximum. However, when cysteine is present in large excess, the intensity slowly increases beyond the anticipated value. In such cases the rate of color development is markedly slower than when the reagent is in excess.

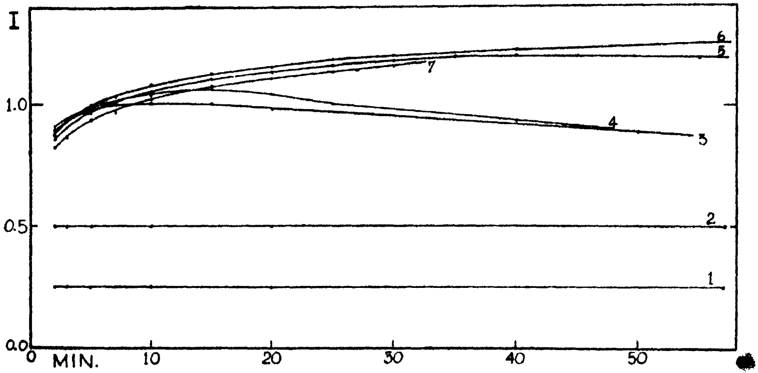

FIG. 2. Color development by A-phospho-18-tungstic acid and cysteine (25° ± 3.0°). Composition of the color solutions: 4 × 10^{-4} M A-phospho-18-tungstic acid and the following concentrations of cysteine (× 10^{-4} M). Curve 1, 1.0; Curve 2, 2.0; Curve 3, 4.0; Curve 4, 5.0; Curve 5, 6.0; Curve 6, 7.0; Curve 7, 10.0. Medium, 0.4 M sodium acetate and 0.12 M acetic acid. Color standard, 4 × 10^{-4} M cysteine with 4 cc. per 50 cc. of the reagent solution. *I* represents color intensity.

In Fig. 2 it is seen that similar relations hold with the free A-phospho-18-tungstic acid as with the ammonium salt.

Fig. 3 shows the results of color production by 0.5 and 2.0 cc. of the A-phospho-18-tungstic acid reagent per 50 cc. with varying amounts of cysteine. By analysis it was found that the reagent solution contains 0.287 gm. of WO_3, which corresponds to 0.016

mole per liter of WO_3. The figure indicates that 0.08×10^{-4} mole of A-phospho-18-tungstic acid produces color which is proportional to cysteine concentration until the latter reaches 0.08×10^{-4} mole (0.8 cc. of 0.01 M cysteine solution in 50 cc. of reaction mixture), beyond which there is practically no rapid increase in color intensity with increase in cysteine concentration. In the other group of curves 0.32×10^{-4} mole of cysteine is the minimum amount that produces practically the maximum color intensity with 0.32×10^{-4} mole of free acid (2 cc. of reagent solution per 50 cc. of mixture).

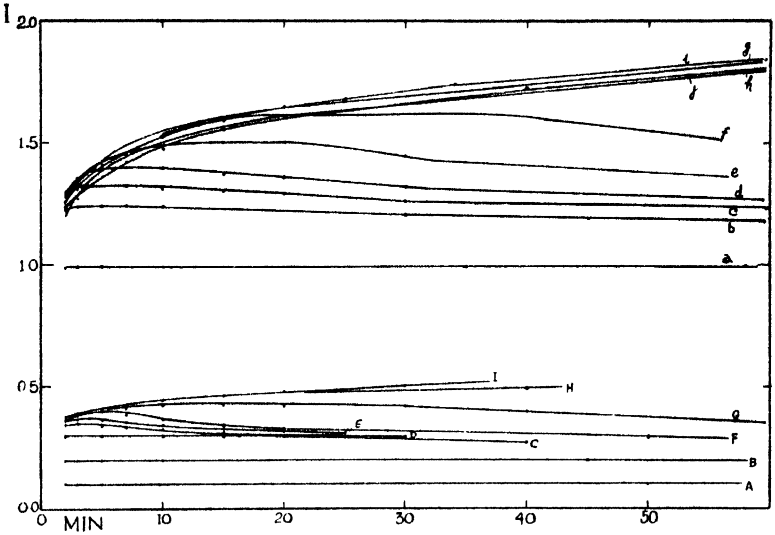

FIG. 3. Color development by the A-phospho-18-tungstic acid reagent solution and cysteine (25° ± 3.0°). Composition of the color solutions: 10 cc. per liter of the reagent solution and the following concentrations of cysteine ($\times 10^{-4}$ M). Curve A, 0.4; Curve B, 0.8; Curve C, 1.2; Curve D, 1.4; Curve E, 1.5; Curve F, 1.6; Curve G, 2.0; Curve H, 4.0; Curve I, 10.0. Curves *a* to *j*, 40 cc. per liter of the reagent solution and the following concentrations of cysteine ($\times 10^{-4}$ M). Curve *a*, 4.0; Curve *b*, 5.0; Curve *c*, 5.2; Curve *d*, 5.6; Curve *e*, 6.0; Curve *f*, 6.4; Curve *g*, 7.0; Curve *h*, 8.0; Curve *i*, 10.0; Curve *j*, 20.0. Medium, 0.4 M sodium acetate and 0.12 M acetic acid. Color standard, 4×10^{-4} M cysteine with 4 cc. per 50 cc. of the reagent solution. *I* represents color intensity.

In experiments with ferrous sulfate and the ammonium salt or the free acid the color produced fades very rapidly, particularly when water is added to make the total volume 50 cc., thus making the determination of the mole ratio impossible. Presence of phosphoric acid in excess seems to be necessary to stabilize the color in this case. Thus, the color produced with the reagent solution is stable, but on account of the precipitation of the basic acetate of iron the intensity determination is impracticable.

DISCUSSION

The above experimental results indicate the general inapplicability of the equation quoted in the first part of this paper. The following explanation is suggested: In the presence of excess of reagent the A-phospho-18-tungstic acid loses 1 equivalent of oxygen per molecule of cysteine, which is thereby converted to cystine. When, however, cysteine is in excess, a 2nd equivalent of oxygen is given up by the complex acid in a slow reaction. The blue compound obtained by Wu (5) is probably produced by this second step, which apparently takes place more rapidly in an alkaline medium. As shown by the author (1), even the first step is markedly retarded with decrease in the pH of the medium below 4.7. Moreover, the equilibrium point of the oxidation-reduction system seems to shift with change in pH.

The assumption that the cysteine is converted to cystine in the above reactions is supported by the observation ((3) p. 683) that the color intensities developed by cysteine are doubled by the addition of sulfite.

Although establishment of an exact chemical equation representing the reaction between cysteine and A-phospho-18-tungstic acid awaits the studies of the oxidation-reduction potential of the complex acid at various pH levels, the following equation may be assumed as representing merely the stoichiometrical relation in the neighborhood of pH 5.

$$2R\text{—}SH + 2H_6P_2O_8(WO_3)_{18} = R\text{—}S\text{—}S\text{—}R + H_2O + [H_6P_2O_8(WO_3)_{17}]_2W_2O_5$$

SUMMARY

A-phospho-18-tungstic acid or its ammonium salt reacts with cysteine at pH 5.0 in equimolar ratio in a first step; in presence of

excess cysteine a second reaction takes place whereby a 2nd equivalent of oxygen is slowly removed from the product of the first step.

BIBLIOGRAPHY

1. Shinohara, K., *J. Biol. Chem.*, **109**, 665 (1935).
2. Shinohara, K., *J. Biol. Chem* , **110**, 263 (1935).
3. Shinohara, K., *J. Biol. Chem* , **112**, 671, 683 (1935–36).
4. Shinohara, K., and Padis, K. E., *J. Biol. Chem.*, **112**, 697 (1935–36).
5. Wu, H., *J. Biol. Chem.*, **43**, 189 (1920).
6. Lugg, J. W. H., *Biochem. J.*, **26**, 2144 (1932).

THE SOURCE OF THE FORMIC ACID PRODUCED ON ACID HYDROLYSIS OF NUCLEIC ACIDS*

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In 1894 Kossel and Neumann (3) isolated and identified levulinic and formic acids produced by acid hydrolysis of thymonucleic acid. The simultaneous formation of these two acids from thymonucleic acid was interpreted by Kossel, and by others who have worked on nucleic acid, as indicating the probable presence in the nucleic acid of a hexose of an unstable nature. This conclusion was based on the fact that hexoses when heated with acid also yield levulinic and formic acids. Brown and Johnson (1) likewise obtained levulinic and formic acids on acid hydrolysis of the nucleic acid of the tubercle bacillus, and they also concluded that the sugar present was a hexose.

Recently Levene and his collaborators (6–8) isolated 2-desoxy-*d*-ribose from thymonucleic acid. They showed that this desoxyribose passed readily into levulinic acid, thus accounting for the levulinic acid appearing on acid hydrolysis of thymonucleic acid, but leaving unexplained the formic acid simultaneously formed, the appearance of which, together with levulinic acid, was supposed to be characteristic of a hexose.

The following investigation was undertaken to determine the amount of formic acid produced and its origin in thymonucleic acid. This work has shown that acid hydrolysis of thymonucleic acid prepared from calf thymus yielded an amount of formic acid which was far less than would be obtained by acid hydrolysis of any hexose examined under similar conditions, and which corresponded approximately to the amount derived from the adenine present in the nucleic acid. The conclusion is, therefore, that little if any of the formic acid originates from the carbohydrate but comes from the adenine chiefly.

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* This is a reprint in part of the dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1937.

EXPERIMENTAL

Hydrolysis and formic acid recovery and determination were carried out by the method of the Association of Official Agricultural Chemists for formic acid determination (9), but the samples of material were generally placed in 20 ml. of 30 per cent (by volume) sulfuric acid rather than 50 ml. of 2 per cent tartaric acid, with exceptions as noted in Table I. In this method the formic acid produced by the action of acid on the substance studied was continuously steam-distilled into a constantly boiling suspension of barium carbonate. Volatile acids, such as formic acid, were caught here. The filtrate from the barium carbonate was treated with reagents containing mercuric chloride, and the HgCl produced by reduction was filtered off and weighed. Any volatile acid in the filtrate capable of reducing mercuric chloride under these conditions was estimated as formic acid. When determinations were run more than 12 hours, they were allowed to stand overnight at room temperature and the distillation resumed on the following day.

To test the method of formic acid determination, controls were run with sodium formate in (*a*) 2 per cent tartaric acid solution, (*b*) 30 per cent sulfuric acid solution, and (*c*) 65 per cent sulfuric acid solution. Blank analyses were carried out with (*a*) and (*b*) alone. The results (Table I) showed that with 2 per cent tartaric or 30 per cent sulfuric acid about 85 to 95 per cent of the formic acid added could be recovered, but with 65 per cent sulfuric acid only 22 per cent was recovered as formic acid, the remainder probably being largely converted to CO and water. The blank analyses were run with 20 ml. of 2 per cent tartaric acid solution alone for 24 hours with 350 ml. of water distilling through the apparatus per hour and yielded 0.2 and 1.0 mg. of HgCl in two determinations. Blank analyses run the same way but with 30 per cent sulfuric acid instead of 2 per cent tartaric acid yielded 1.8 and 0.7 mg. of HgCl in two determinations.

To test the interference of any levulinic acid, formed in the hydrolyses, with the formic acid determination, controls were run with levulinic acid (Eastman) in (*a*) 2 per cent tartaric acid solution, (*b*) 5 per cent tartaric acid solution, and (*c*) 30 per cent sulfuric acid solution. 13 to 20 cg. of levulinic acid were placed in the tartaric or sulfuric acid and treated by the usual procedure for 24 hours. The results (Table I) show that the presence of levulinic acid in the acid solution leads to the production of a small percentage of calomel in the final stages of the determination. The calomel precipitate in these cases was yellow in color instead of white. Since levulinic acid added directly to mercuric chloride solution gives a similar yellow precipitate, it is probable that small quantities of levulinic acid passed over in those determinations in which levulinic acid was formed in the hydrolysis. The amount thus passing over to contaminate the formic acid determination was, however, so small as to introduce no serious error. In hydrolyses of adenine and guanine the calomel precipitate was always white, indicating that no contamination of this kind occurred in these instances.

To test the interference of any sulfurous acid, formed in the hydrolyses, with the formic acid determination, controls were run with sodium sulfite placed in 30 per cent sulfuric acid and treated by the usual procedure for 12 hours. No sulfites or reducing power could be detected in the barium carbonate suspensions. In further control experiments, added sulfite was found to disappear rapidly from boiling suspensions of barium carbonate in water. Hence, any sulfurous acid formed in the course of the hydrolyses did not interfere with the formic acid determinations as carried out in this work.

To measure the production of formic acid from known sugars when hydrolyzed under similar conditions, glucose, galactose, levulose, arabinose, and xylose were heated in 30 per cent sulfuric acid with the usual procedure. It will be seen (Table I) that only small amounts of formic acid were obtained from the pentoses, but 6 to 18 per cent from the hexoses, levulose giving most and glucose least, but the latter still giving about 14 per cent in 24 hours of heating.

To measure the production of formic acid from pyrimidines and purines, certain of these substances were heated with 30 per cent sulfuric acid for similar periods and the formic acid produced determined. Uracil, thymine, cytosine, uric acid, adenine sulfate, and guanine hydrochloride were thus treated.

The uracil was synthesized by Dr. Kuizenga in this laboratory by the method of Davidson and Baudisch (2). It was found to contain 25.5 and 25.1 per cent nitrogen (theory, 25.00 per cent N) in two analyses. The thymine and cytosine were kindly given us by Dr. Heyroth of the Basic Science Laboratory. The adenine sulfate (Eastman) was found to contain 34.6 and 33.5 per cent nitrogen (theory, 34.63 per cent N) in two analyses. The guanine hydrochloride (Eastman) was found to contain 33.6 and 33.7 per cent nitrogen (theory, 34.07 per cent N) in two analyses.

The pyrimidines when heated with 30 per cent sulfuric acid gave minimal amounts, or no formic acid, with the exception of cytosine which gave in one determination about 3 per cent. We believe this anomalous result to be in error but are without explanation for it.

Of the purines, adenine sulfate was especially able to form formic acid, and produced large quantities (25 to 27 per cent) in short periods (24 hours) of treatment. Guanine hydrochloride also yielded a little formic acid (about 1 per cent in 24 hours), while uric acid gave rise practically to none (see Table I).

Sodium thymonucleate was prepared from calf thymus by the Levene and Bass modification (5) of Levene's method (4) and was purified according to Steudel and Peiser (10). It gave negative biuret and Millon's tests, and was found, when dried *in vacuo* at room temperature over P_2O_5, to contain 12.9 and 13.4 per cent nitrogen (theory, 15.66 per cent N) in two analyses, and 7.55 and 7.76 per cent phosphorus (theory, 9.25 per cent P) in two analyses. The nitrogen to phosphorus ratio found was 1.7 (theory, 1.69).

Yeast nucleic acid (Pfanstiehl), similarly dried, was found to contain 13.9 and 14.8 per cent nitrogen (theory, 16.12 per cent N) in two analyses, and 7.44 and 7.48 per cent phosphorus (theory, 9.53 per cent P) in two analyses. The N:P ratio found was 1.9 (theory 1.69).

The sodium thymonucleate and the yeast nucleic acid were heated with 30 per cent sulfuric acid for similar periods and the formic acid produced determined (Table I). The average yield from sodium thymonucleate heated with 30 per cent sulfuric acid was 3.2 per cent formic acid in 24 hours of treatment, and from yeast nucleic acid, 3.6 per cent formic acid in 24 hours, approxi-

TABLE I

Average Values Found in Formic Acid Determinations

Material treated	Weight of material	Weight of $HgCl$ minus blank*	Yield of $HCOOH$	Treatment	Acid used†	Distillate per hr.
	gm.	*mg.*	*per cent*	*hrs.*	*per cent*	*ml.*
$HCOONa$	0.0189	122.4	63.6	3	2 T.	300
"	0.0221	136.0	59.5	3	30 S.	400
" ‡	0.0212	32.6	15.0	3	65 "	400
Levulinic acid	0.14	23.5	1.7	24	2 T.	350
" "	0.17	17.2	0.88	24	5 "	185
" "	0.17	26.4	1.6	24	30 S.	160
" "	0.14	23.3	1.6	24	30 "	350
Glucose‡	0.1024	62.8	5.98	3	30 "	400
		116.5	11.09	6	30 "	400
"	0.1506	159.0	10.47	12	30 "	140
		219.6	13.79	24	30 "	155
Galactose‡	0.1024	91.7	8.73	3	30 "	500
		157.4	14.99	6	30 "	435
Levulose	0.1007	189.6	18.4	12	30 "	400
Arabinose	0.5222	24.4	0.45	12	30 "	200
Xylose	0.8412	28.2	0.34	3	30 "	200
		49.0	0.57	12	30 "	235
		50.0	0.59	24	30 "	220
Thymine	0.0356	2.6	0.70	48	30 "	200
Cytosine‡	0.0182	6.3	3.4	24	30 "	200
		10.7	5.7	60	30 "	200
"	0.0182	0.0	0.0	48	30 "	200
Uracil	0.4765	2.3	0.04	12	30 "	260
		4.5	0.08	24	30 "	255
Uric acid	0.6889	2.0	0.03	24	30 "	180
Adenine sulfate	0.4285	1100.5	26.29	24	30 "	210
" "	0.0293	77.0	25.8	24	30 "	200
Guanine hydrochloride	0.4919	62.6	1.24	24	30 "	190
" "	0.0287	16.3	5.60	60	30 "	140
Yeast nucleic acid	0.3790	138.1	3.56	24	30 "	190
Sodium thymonucleate‡	0.2124	31.7	1.46	3	30 "	400
" "	0.2000	70.4	3.41	24	30 "	200
		97.8	4.75	63	30 "	200
" "	0.4034	123.2	2.98	24	30 "	350
		189.4	4.58	48	30 "	350
" "	0.4042	37.4	0.90	3	30 "	270

* The blank amounted to 0.0 to 1.8 mg. of $HgCl$ during the course of the work.

† Results of formic acid determinations on materials heated with 2 per cent tartaric acid (2 T.), 5 per cent tartaric acid (5 T.), 30 per cent sulfuric acid (30 S.), and 65 per cent sulfuric acid (65 S.).

‡ These figures are for single determinations; *i.e.*, are not average figures.

mately the same amount. By calculating the formic acid production of the individual substances, one finds:

1 mole adenine	yielded	52	gm.	HCOOH	in 24 hrs.
1 " guanine	"	2 6	"	"	" 24 "
4 moles xylose	"	3 6	"	"	" 24 "
Total yield =		58	"	"	" 24 "

While the expected yield, calculated on xylose in place of *d*-ribose, is thus 58 gm. of formic acid from 1 mole of yeast nucleic acid, that found was 47 gm. The sodium thymonucleate gave 43 gm. of formic acid per mole. Some of the sugar of the thymonucleate was converted to humus; and the yellow color of the calomel precipitates, when levulinic acid was present in the distilling flask, indicated that an indeterminate but probably very small amount of levulinic acid may have contaminated the formic acid determinations. The differences between the amounts of formic acid expected and found may be due to the different reactivities of the sugars of the nucleic acids from that of xylose used in the calculation, or to the considerable errors of the method.

SUMMARY

The chief source of formic acid obtained by acid hydrolysis of thymonucleic acid is adenine, while guanine contributes a very minor portion.

Yeast nucleic acid also yields formic acid on acid hydrolysis and to about the same amount as does thymonucleic acid. This observation surprised us. The chief source of this formic acid is adenine, while guanine and ribose contribute minor portions.

It is probable that the chief sources of formic acid produced on acid hydrolysis of the nucleic acid of the tubercle bacillus are its adenine, and to a much less extent, guanine radicals.

Since Levene has shown that desoxyribose, on acid hydrolysis, forms levulinic acid, it thus happens that these two substances, levulinic and formic acids, are now accounted for. For 20 years or more the fact that nucleic acids yielded these two substances on acid hydrolysis was considered evidence that the carbohydrate in them was a hexose, since hexoses also show this peculiarity. We now know that the sugar of thymonucleic acid was really a desoxypentose and the formic acid came almost entirely from adenine.

I wish to thank Professor A. P. Mathews for his kind advice and criticism throughout this work, and Dr. F. F. Heyroth of the Basic Science Laboratory for the thymine and cytosine which he kindly gave.

BIBLIOGRAPHY

1. Brown, E., and Johnson, T. B., *J. Am. Chem. Soc.*, **45**, 1823 (1923).
2. Davidson, D., and Baudisch, O., *J. Am. Chem. Soc.*, **48**, 2379 (1926).
3. Kossel, A., and Neumann, A., *Ber. chem. Ges.*, **27**, 2215 (1894).
4. Levene, P. A., *J. Biol. Chem.*, **53**, 441 (1922).
5. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 299 (1931).
6. Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929).
7. Levene, P. A., Mikeska, L. A., and Mori, T., *J. Biol. Chem.*, **85**, 785 (1929–30).
8. Levene, P. A., and Mori, T., *J. Biol. Chem.*, **83**, 803 (1929).
9. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 3rd edition (1930).
10. Steudel, H., and Peiser, E., in Klein, G., Handbuch der Pflanzenanalyse, Vienna, **4**, 435 (1933).

ROTATORY DISPERSION OF CONFIGURATIONALLY RELATED AMINES

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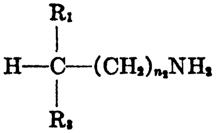
The present communication deals with three points regarding the configurational relationships of aliphatic amines of the general type



$$\begin{array}{c} R_1 \\ | \\ H—C—(CH_2)_{n_2}NH_2 \\ | \\ R_2 \end{array}$$

The first is concerned with the direction of rotation in correlated secondary amines ($n_2 = 0$) and primary amines ($n_2 > 0$); the second with the direction of rotation in the members of the series of secondary amines homologous with respect to R_1; the third with the comparison of the dispersion curves of the amines and those of the corresponding alcohols.

The correlation of the configurations of primary and secondary amines can be arrived at by arguments identical with those employed in the case of alcohols;[1] namely, by comparing the direction of rotation in the accompanying four amines (I to IV).

In the three amines (I), (III), and (IV), the substituents are arranged counter-clockwise;[2] in (II) the order is reversed. Inasmuch as it is generally accepted that in hydrocarbons, alcohols, and amines the direction of rotation is determined by the clock-

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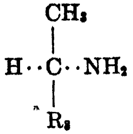
[1] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **116**, 209 (1936).

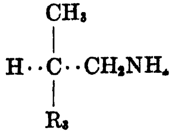
[2] The abbreviated expression "clockwise or counter-clockwise" is used in the following sense. When a molecule is viewed with the largest group toward the observer, the three other groups are arranged in clockwise or counter-clockwise order according to diminishing volume.



$$\begin{array}{c} CH_3 \\ | \\ H\cdot\cdot C\cdot\cdot NH_2 \\ | \\ R_3 \end{array}$$

Dextro

I

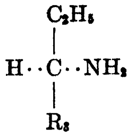


$$\begin{array}{c} CH_3 \\ | \\ H\cdot\cdot C\cdot\cdot CH_2NH_2 \\ | \\ R_3 \end{array}$$

Levo

II

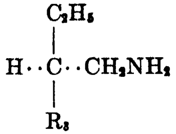
$(R_3 > C_2H_5 > NH_2)$



$$\begin{array}{c} C_2H_5 \\ | \\ H\cdot\cdot C\cdot\cdot NH_2 \\ | \\ R_3 \end{array}$$

Dextro

III



$$\begin{array}{c} C_2H_5 \\ | \\ H\cdot\cdot C\cdot\cdot CH_2NH_2 \\ | \\ R_3 \end{array}$$

Dextro

IV

wise or counter-clockwise arrangement of the substituents, it follows that substances (I), (III), and (IV) should be of one sign and substance (II) of the other.

In order to justify this conclusion it was necessary to show that the rotatory dispersions of substances (II) and (IV) are both normal. From Table V it can be seen that this is the case.

The direction of rotation in members of the series of secondary amines homologous with respect to R_1 was unsettled, since it was observed that the members of the corresponding series of azides, from which the amines were derived by reduction, differed in sign in members having $R_1 = CH_3$ or C_2H_5 and in those having $R_1 > C_2H_5$, even though all the azides were obtained from iodides having the same sign of rotation and similar rotatory dispersion.

Analysis of the dispersion curves of the amines, however, did not give a clear cut answer, as will be seen from the discussion in the experimental part. The difficulty arose from the fact that the nearest to the visible absorption regions of the amines are not anisotropic. It was therefore decided to study such derivatives of the amines as would have absorption bands nearer to the visible region. The benzoyl derivatives were chosen for this purpose. Analysis of the latter indicates that in the series of secondary amines homologous with respect to R_1 all members rotate in the same direction.

Rotatory Dispersion of Primary Amines—It was found that the direction of rotation of primary amines is of the same sign as that of the corresponding alcohols. It has been found that corresponding rotatory dispersions likewise are similar. As in the alcohols, so in the amines, the absorption bands nearest to the visible region are not anisotropic.

Inasmuch as the configurational relationships between secondary and primary amines are similar to those of primary and secondary alcohols, it may be justified to conclude that amines, as a rule, rotate in the same direction as the corresponding alcohols.

SUMMARY

1. The correlation of the configuration of primary and secondary amines is similar to that of the primary and secondary alcohols.
2. The direction of rotation of primary and of secondary amines is identical with that of corresponding alcohols.
3. In all alkyl amines the absorption regions nearest to the visible region are not anisotropic.

EXPERIMENTAL

Absorption Spectra—The absorption curves of many simple secondary and primary amines have recently been determined.[3] For all of them the absorption starts from $\simeq \lambda$ 2400 and increases very rapidly for shorter wave-lengths ($\epsilon_{2300} \simeq 50$ in hexane solution). The absorption curves of the hydrochlorides are displaced towards higher frequencies by about 200 Å.

Rotatory Dispersion Curves—Rotatory dispersion data are summarized in Tables II to V. It appears that the first absorption region, λ 2400 to λ 2000, is not anisotropic. This is in accord with what was found for so many other substances. Contrary to the rule of Kuhn, the first absorption region of substances with one chromophoric group is as a rule inactive, aliphatic aldehydes being an exception.

Secondary Amines—It may be seen from Table I that all secondary free amines homologous with respect to R_1 have the same sign of rotation. Identity of direction of rotation in members of homologous series generally implies identity of sign of corresponding partial rotations, since in numerous cases it was found that *the partial rotation of a band of the functional group has the*

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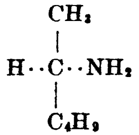
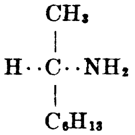
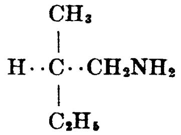
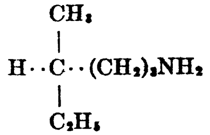
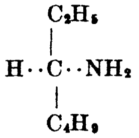
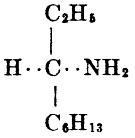
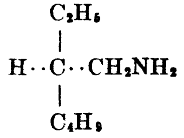
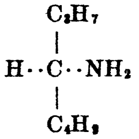
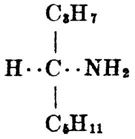
[3] Grunfeld, M., *Ann. chim.*, series 10, **20**, 304 (1933).

TABLE I

Configurationally Related Amines $[M]^{25}_{5780}$ (*Approximate Maximum Values*)

 $\begin{array}{c} CH_3 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_4H_9 \end{array}$ Free amine +4 8° HCl −2 9° Benzoyl +31 4°	 $\begin{array}{c} CH_3 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_6H_{13} \end{array}$ Free amine +6 8° HCl −10 2°	 $\begin{array}{c} CH_3 \\ \vert \\ H\cdot\cdot C\cdot\cdot CH_2NH_2 \\ \vert \\ C_2H_5 \end{array}$ Free amine −5 2°	 $\begin{array}{c} CH_3 \\ \vert \\ H\cdot\cdot C\cdot\cdot (CH_2)_3NH_2 \\ \vert \\ C_2H_5 \end{array}$ Free amine +12 4° HCl +13 9°
 $\begin{array}{c} C_2H_5 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_4H_9 \end{array}$ Free amine +4 2° HCl +1 7° Benzoyl +6 1°	 $\begin{array}{c} C_2H_5 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_6H_{13} \end{array}$ Free amine +7 2° HCl + or − Benzoyl +13 9°	 $\begin{array}{c} C_2H_5 \\ \vert \\ H\cdot\cdot C\cdot\cdot CH_2NH_2 \\ \vert \\ C_4H_9 \end{array}$ Free amine +3 2° HCl +8 4°	
 $\begin{array}{c} C_3H_7 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_4H_9 \end{array}$ Free amine +0 6° HCl −1 9° Benzoyl +2 2°	 $\begin{array}{c} C_3H_7 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ C_5H_{11} \end{array}$ Free amine +0 7° HCl −1 5°		

same sign in all members of homologous series (it being understood that for all members considered $R_1 > R_3$ or $R_3 > R_1$).

The results of the analysis of the rotatory dispersion curves of the secondary free amines homologous with respect to R_1 seem to contradict that rule (Tables II and III). Thus, the dispersion constant of 2-amino-octane is low ($\lambda_0^2 = 0.0185$ for a one Drude term formula) and measurements in the ultraviolet region indicate that the dispersion is anomalous. On the other hand, the dispersion constant of 4-aminooctane or 4-aminononane is high ($\lambda_0^2 = 0.042$ or 0.050 respectively), indicating that the dispersion is normal and should be expressed by two terms of opposite sign. It may be that the system of optically active absorption bands in amines is of such complexity that closely located absorption bands anisotropic when $R_1 = CH_3$ and inactive when $R_1 = C_3H_7$, or *vice versa,* are the cause of these anomalies. It is perhaps significant that the value of λ_0^2 increases progressively in members of the series homologous with respect to R_1.

The difficulty encountered in the interpretation of the dispersion of the free amines is met also in the case of the hydrochlorides, as appears from Tables II and III. All hydrochlorides are opposite in sign to the free amines with the exception of 3-aminoheptane It should be noticed that hydrochlorides of the amines having $R_1 = CH_3$ are more dispersive than the free amines and *vice versa* for the substances having $R_3 = C_3H_7$. As seen from Table III, the rotation of the hydrochloride of 3-aminononane can be of either sign, depending upon concentration. This offers no difficulty since in one case the dispersion is anomalous and normal in the other.

No difficulty of interpretation is encountered in the benzoyl derivatives (see Table IV). All of them have the same sign of rotation and their rotatory dispersion is normal.

The dispersion constant of the benzoyl derivative of 4-aminononane is especially high, undoubtedly because the absolute magnitudes of the first two opposite contributions are closer than in the other benzoyl derivatives; hence the low values for the molecular rotation.

Primary Amines—The results in the primary amines parallel entirely those observed in the corresponding alcohols. As can be seen from Table V, the amine with $n_2 = 1$ has a high dispersive

TABLE II

Rotatory Dispersion of Configurationally Related Secondary Amines in Homogeneous State and Their Hydrochlorides in Aqueous Solution

λ	Dextro-2-aminohexane $d_4^{25} = 0.755$ (*in vacuo*)				Dextro-2-aminooctane $d_4^{25} = 0.771$; $n_D^{25} = 1.4220$				Dextro-3-aminoheptane $d_4^{25} = 0.7628$ (*in vacuo*); $n_D^{25} = 1.4163$			
	Free amine $l = 5$ cm. $[M]^{25}_{max.} = \frac{1.514}{\lambda^2 - 0.019}$		Hydrochloride $c = 1.66$ M; $l = 20$ cm. $[M]^{25}_{max.} = -\frac{0.889}{\lambda^2 - 0.024}$		Free amine $l = 10$ cm.; ultraviolet $l = 5$ cm. $[M]^{25}_{max} = \frac{2.155}{\lambda^2 - 0.0185}$		Hydrochloride $c = 0.881$ M; $l = 20$ cm.; ultraviolet $l = 5$ cm. $[M]^{25}_{max} = -\frac{3.170}{\lambda^2 - 0.0248}$		Free amine $l = 20$ cm.; ultraviolet $l = 5$ cm. $[M]^{25}_{max.} = \frac{1.279}{\lambda^2 - 0.028}$		Hydrochloride $c = 1.61$ M; $l = 40$ cm.; ultraviolet $l = 10$ cm. $[M]^{25}_{max.} = \frac{5.282}{\lambda^2 - 0.032}$	
	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$
5892.6					3.233	6.58	−1.434	−9.89				
5875.6	1.605	4.67	−0.861	−2.82					3.287	4.020	0.676	1.702
5780.1	1.651	4.80	−0.877	−2.87	3 355	6.828	−1.487	−10.25	3.417	4.179	0.695	1.750
5460.7	1.857	5.40	−0.989	−3.24	3.797	7.728	−1.689	−11.65	3.872	4.735	0.790	1.989
4358.3	3.04	8.85	−1.64	−5 36	6.19_6	12.61	−2.79	−19.24	6.46	7.90	1 33	3.35
4046.6	3.47	10.1			7.29	14.84	−3 31	22.82	7.69	9.40	1 57	3.95
3920					3 90	15 9						
3560									2.60	12.7		
3340									3.10	15.2		
3250					−5 90	24 0			3.60	17.6		
3180												
3100									3.85	18 8		
3060							−1 65	−45 5				
3040					−6 90	28 1						
3020									4.10	20.0		

3000											0.9	9.1
2970									4.35	21.3		
2920									4 60	22 5		
2880											1.04	10.0
2830							−2 15	−59 3				
2750					−0 87 (0.5 cm.)	35 4						
2730							−2.40	−66 2				
2650							−2 65	−73.1				
2500					−0.87 (0.5 cm.)	35.4						
2470							−3 15	−86.9				
2370							−3 65	−101				

TABLE III

Rotatory Dispersion of Configurationally Related Secondary Amines in Homogeneous State and Their Hydrochlorides in Aqueous Solution

λ	Dextro-3-aminononane						Dextro-4-aminooctane $d_4^{25} = 0.7702$ (*in vacuo*)				Dextro-4-aminononane $d_4^{25} = 0.7772$ (*in vacuo*)			
	Free amine		Hydrochloride				Free amine		Hydrochloride		Free amine		Hydrochloride	
	$l = 10$ cm.		$c = 1.134$ M; $l = 20$ cm.		$c = 0.283$ M; $l = 40$ cm.		$l = 20$ cm.		$c = 2.17$ M; $l = 20$ cm.		$l = 10$ cm.		$c = 1.05$ M; $l = 40$ cm.	
	$[M]^{25}_{\text{max.}} = \frac{2.209}{\lambda^2 - 0.027}$		$[M]^{25}_{\text{max.}} = -\frac{0.285}{\lambda^2}$		$[M]^{25}_{\text{max.}} = \frac{1.327}{\lambda^2 - 0.037}$		$[M]^{25}_{\text{max.}} = \frac{0.172}{\lambda^2 - 0.0424}$		$[M]^{25}_{\text{max.}} = -\frac{0.579}{\lambda^2 - 0.029}$		$[M]^{25} = \frac{0.187}{\lambda^2 - 0.050}$		$[M]^{25} = -\frac{0.460}{\lambda^2 - 0.026}$	
	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}$	^{25}	$[M]^{25}$
5875.6	3.571	6.963			0.473	4.36	0.340	0.570					−0.605	−1.44
5780.1	3.694	7.203	−0.185	−0.853	0.484	4.46	0.351	0.588	−0.822	−1.895	0.358	0.659	−0.629	−1.50
5460.7	4.182	8.155	−0.207	−0.954	0.550	5.07	0.401	0.672	−0.920	−2.122	0.405	0.746	−0.709	−1.69
4358.3	6.97	13.59	−0.325	−1.50	0.94	8.66	0.695	1.16	−1.56	−3.60	0.73	1.34	−1.18	−2.81
4046.6	8.31	16.20	−0.37	−1.70	1.13	10.4	0.835	1.40	−1.85	−4.26	0.93	1.71	−1.39	−3.32

power, the dispersion constant of a single Drude term $\lambda_0^2 = 0.0585$ being too high to correspond to the position of the first active absorption band. Consequently the rotatory power is the sum of two terms of opposite sign whose magnitudes are of the same order. This is in harmony with the low absolute value of the molecular rotation.

The amine with $n_2 = 3$ is less dispersive and the value of the dispersion constant $\lambda_0^2 = 0.022$ is about the same as that found for the corresponding carbinol.

A similarity in the value of the molecular rotations corresponds to this similarity in the dispersive power of amines and alcohols. It thus shows that the analogy in the system of optically active absorption bands must be in fact very close in both types of compounds. For instance, for 2-methyl-1-aminobutane, $[M]^{25}_{5876} = -5.1°$, and for the corresponding alcohol, 2-methylbutanol-1, $[M]^{25}_{5876} = -5.2°$; for 4-methyl-1-aminohexane, $[M]^{25}_{5876} = 12.0°$, and for the corresponding alcohol, 4-methylhexanol-1, $[M]^{25}_{5876} = 11.9°$.

TABLE IV

Rotatory Dispersion of Benzoyl Derivatives of Configurationally Related Secondary Amines

λ	Dextro-benzoyl of dextro-2-aminohexane in alcohol		Dextro-benzoyl of dextro-3-aminononane in alcohol		Dextro-benzoyl of dextro-4-aminooctane in alcohol	
	$c = 0.421$ M; $l = 20$ cm. $[M]^{25}_{\text{max.}} = \frac{9.600}{\lambda^2 - 0.0404}$		$c = 0.532$ M; $l = 20$ cm. $[M]^{25}_{\text{max.}} = \frac{4.198}{\lambda^2 - 0.033}$		$c = 0.776$ M; $l = 20$ cm. $[M]^{25}_{\text{max.}} = \frac{0.567}{\lambda^2 - 0.076}$	
	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$
5875.6	2.474	31.44				
5780.1	2.572	32.69	1 425	13.93	0.335	2.16
5460.7	2.934	37.29	1 620	15.83	0 395	2.54
4358.3	5.05	64.2	2 73	26.70	0 75	4.83
4046.6	6.13	77.8				

Preparation

Dextro-Benzoyl-2-Aminobutane—2 gm. of 2-aminobutane hydrochloride, $[M]^{25}_D = -0.44°$ (in water),[4] were converted into the benzoyl derivative by the method of Schotten and Baumann. It

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[4] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

TABLE V

Rotatory Dispersion of Configurationally Related Primary Amines in Homogeneous State and Their Corresponding Hydrochlorides in Aqueous Solution

λ	Levo-2-methyl-1-aminobutane $d_4^{25} = 0.750$ (*in vacuo*)		Dextro-4-methyl-1-aminohexane $d_4^{25} = 0.7709$ (*in vacuo*); $n_D^{25} = 1.4220_8$				Dextro-2-ethyl-1-aminohexane $d_4^{25} = 0.7844$ (*in vacuo*); $n_D^{25} = 1.4286$			
	Free amine $l = 5$ cm. $[M]^{25}_{max} = -\frac{1.441}{\lambda^2 - 0.0585}$		Free amine $l = 20$ cm.; ultraviolet $l = 5$ cm. $[M]^{25}_{max.} = \frac{3.876}{\lambda^2 - 0.0222}$		Hydrochloride $c = 1.412$ M; $l = 40$ cm. $[M]^{25}_{max.} = \frac{4.335}{\lambda^2 - 0.0232}$		Free amine $l = 20$ cm. $[M]^{25}_{max.} = \frac{0.974}{\lambda^2 - 0.028}$		Hydrochloride $c = 1.983$ M; $l = 40$ cm. $[M]_{max.} = \frac{2.506}{\lambda^2 - 0.0381}$	
	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$
5875.6	−0.923	−5.10	5.755	12.00	2.744 (?)	13.58	0.808	3.05	1.405	8.15
5780.1	−0.953	−5.26	5.957	12.42	2.813	13.92	0.845	3.19	1.460	8.47
5460.7	−1.087	−6.00	6.735	14.04	3.184	15.76	0.955	3.60	1.660	9.63
4358.3	−1.99	−11.0	11.08	23.10	5.25	25.98	1.595	6.02	2.84	16.50
4046.6	−2.42	−13.4	13.13	27.38	6.23	30.83	1.90	7.17	3.44	19.95
3740			3.90	32.5						
3590			4.40	36.7						
3420			4.90	40.9						
3300			5.40	45.0						
3170			5.90	49.2						
3080			6.40	53.4						
2995			6.90	57.5						
2835			7.90	65.9						
2760			8.40	70.0						
2530			1.0 (0.5 cm.)	83.4						
2490			1.1 (0.5 ")	91.7						

was recrystallized twice by dissolving it in ether and then adding pentane until crystals appeared. M.p. 86–88°.

$$[\alpha]^{25}_{D} = \frac{+0.40^\circ \times 100}{1 \times 6.0} = +6.7^\circ; \quad [M]^{25}_{D} = +11.8^\circ \text{ (in absolute alcohol)}$$

4.706 mg. substance: 12.915 mg. CO_2 and 3.580 mg. H_2O
$C_{11}H_{15}ON$. Calculated. C 74.52, H 8.54
177.1 Found. " 74.83, " 8.51

Dextro-Benzoyl-2-Aminooctane—0.8 gm. of 2-aminooctane hydrochloride

$$[\alpha]^{25}_{D} = \frac{-0.55^\circ \times 100}{1 \times 10.0} = -5.5^\circ \text{ (in water)}^4$$

were converted into the benzoyl derivative. M.p. 73–74°.

$$[\alpha]^{25}_{D} = \frac{+2.85^\circ \times 100}{1 \times 10.0} = +28.5^\circ; \quad [M]^{25}_{D} = +66.2^\circ \text{ (in absolute alcohol)}$$

3.376 mg. substance: 9.547 mg. CO_2 and 3.005 mg. H_2O
$C_{15}H_{22}ON$. Calculated. C 77.52, H 9.55
232.2 Found. " 77.11, " 9.96

Dextro-2-Hexanol—2-Hexanol was prepared from *n*-butyl magnesium bromide and acetaldehyde. The carbinol was resolved as described by Pickard and Kenyon.[5] The insoluble strychnine salt gave a carbinol which boiled at 99–100°, p = 168 mm.

$$[\alpha]^{25}_{D} = \frac{+8.72^\circ}{1 \times 0.813} = +10.7^\circ; \quad [M]^{25}_{D} = +10.9^\circ \text{ (homogeneous)}$$

3.304 mg. substance: 8.535 mg. CO_2 and 4.080 mg. H_2O
$C_6H_{14}O$. Calculated. C 70.51, H 13.82
102.1 Found. " 70.44, " 13.81

Levo-2-Iodohexane—20 gm. of 2-hexanol, $[\alpha]^{25}_{D} = +10.7^\circ$ (homogeneous), were treated with anhydrous hydrogen iodide as described.[4] The sealed bomb tube was allowed to stand at room temperature overnight. The iodide boiled at 90–91°, $p = 70$ mm. Yield 38 gm.; $n_D^{25} = 1.4911$.

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[5] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, **99**, 58 (1911).

$$[\alpha]_D^{25} = \frac{-43.6°}{1 \times 1.42} = -30.7°; \qquad [M]_D^{25} = -65.1° \text{ (homogeneous)}$$

3.610 mg. substance: 4.490 mg. CO_2 and 2.005 mg. H_2O
$C_6H_{13}I$. Calculated. C 33.96, H 6.18
212.0 Found. " 33.89, " 6.21

Dextro-2-Azidohexane—38 gm. of 2-iodohexane, $[\alpha]_D^{25} = -30.7°$ (homogeneous), were added to a solution of 14 gm. of sodium azide (Kahlbaum) in 38 cc. of water and 400 cc. of methanol. This was sealed into four bomb tubes and heated at 80° for 16 hours. The azide was isolated as usual.[4] B.p. 96–98°, $p = 160$ mm. Yield 17 gm.; $d_4^{25} = 0.8571$ (*in vacuo*); $n_D^{25} = 1.4253$.

$$[\alpha]_D^{25} = \frac{+23.85°}{1 \times 0.857} = +27.8°; \qquad [M]_D^{25} = +35.3° \text{ (homogeneous)}$$

3.515 mg. substance: 7.320 mg. CO_2 and 3.310 mg. H_2O
$C_6H_{13}N_3$. Calculated. C 56.64, H 10.31
127.1 Found. " 56.79, " 10.53

Levo-2-Aminohexane—17 gm. of 2-azidohexane, $[\alpha]_D^{25} = +27.8°$ (homogeneous), were dissolved in 35 cc. of methanol and 0.7 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The amine hydrochloride was isolated as usual.[6] Yield 7.5 gm.

$$[\alpha]_D^{25} = \frac{-2.27° \times 100}{2 \times 20.0} = -5.68°; \qquad [M]_D^{25} = -7.82° \text{ (absolute alcohol)}$$

4.151 mg. substance: 8.000 mg. CO_2 and 4.380 mg. H_2O
$C_6H_{16}NCl$. Calculated. C 52.33, H 11.72
137.6 Found. " 52.55, " 11.80

The salt was converted into the free base as usual. B.p. 70°, $p = 155$ mm. $d_4^{27} = 0.755$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+3.25°}{1 \times 0.755} = +4.30°; \qquad [M]_D^{25} = +4.35° \text{ (homogeneous)}$$

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[6] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **115**, 267 (1936).

Dextro-Benzoyl-2-Aminohexane—1.5 gm. of 2-aminohexane hydrochloride, $[\alpha]_D^{25} = -5.68°$ (absolute alcohol), were converted into the benzoyl derivative. M.p. 86–88°.

$$[\alpha]_D^{25} = \frac{+2.85° \times 100}{1 \times 20.0} = +14.3°; \qquad [M]_D^{25} = +29.3° \text{ (in absolute alcohol)}$$

3.982 mg. substance: 11.080 mg. CO_2 and 3.325 mg. H_2O
$C_{13}H_{19}ON$. Calculated. C 76.04, H 9.33
205.2 Found. " 75.89, " 9.34

Dextro-3-Heptanol—The inactive carbinol was prepared from *n*-butyl magnesium bromide and propionaldehyde as usual. It was resolved according to the procedure of Pickard and Kenyon.[7] The less soluble salt gave a carbinol which boiled at 104–106°, $p = 117$ mm.

$$[\alpha]_D^{25} = \frac{+4.20°}{1 \times 0.820} = +5.12°; \qquad [M]_D^{25} = +5.94° \text{ (homogeneous)}$$

4.320 mg. substance: 11.483 mg. CO_2 and 5.375 mg. H_2O
$C_7H_{16}O$. Calculated. C 72.33, H 13.89
116.1 Found. " 72.48, " 13.92

Levo-3-Iodoheptane—60 gm. of 3-heptanol, $[\alpha]_D^{25} = +5.12°$ (homogeneous), were treated with anhydrous hydrogen iodide as usual. The three sealed bomb tubes were allowed to stand overnight at room temperature. The iodide distilled at 76°, $p = 12$ mm. Yield 78 gm.; $d_4^{25} = 1.3675$ (*in vacuo*); $n_D^{25} = 1.4901$.

$$[\alpha]_D^{25} = \frac{-11.3°}{1 \times 1.37} = -8.25°; \qquad [M]_D^{25} = -18.6° \text{ (homogeneous)}$$

4.916 mg. substance: 6.695 mg. CO_2 and 2.905 mg. H_2O
$C_7H_{15}I$. Calculated. C 37.16, H 6.69
226.0 Found. " 37.13, " 6.61

Dextro-3-Azidoheptane—78 gm. of 3-iodoheptane, $[\alpha]_D^{25} = -8.25°$ (homogeneous), were added to a solution of 30 gm. of sodium azide (Kahlbaum) in 70 cc. of water and 750 cc. of methanol. The solution was sealed into nine bomb tubes and heated for 14 hours at 75°. The azide was isolated as usual. B.p. 79–81°, $p = 43$ mm. Yield 13 gm.; $d_4^{25} = 0.8583$ (*in vacuo*); $n_D^{25} = 1.4298$.

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[7] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, **103**, 1944 (1913).

$$[\alpha]_D^{25} = \frac{+1.53^\circ}{1 \times 0.858} = +1.78^\circ; \quad [M]_D^{25} = +2.51^\circ \text{ (homogeneous)}$$

3.115 mg. substance: 6.805 mg. CO_2 and 2.960 mg. H_2O
$C_7H_{15}N_3$. Calculated. C 59.52, H 10.71
141.1 Found. " 59.57, " 10.63

Dextro-3-Aminoheptane—13 gm. of 3-azidoheptane, $[\alpha]_D^{25} = +1.78^\circ$ (homogeneous), were dissolved in 40 cc. of methanol, and 0.5 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 3 hours. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{+0.18^\circ \times 100}{1 \times 18.0} = +1.00^\circ; \quad [M]_D^{25} = +1.52^\circ \text{ (10\% HCl)}$$

The free base was isolated from the salt. B.p. 75°, $p = 70$ mm. $d_4^{25} = 0.783$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+3.25^\circ}{1 \times 0.783} = +4.15^\circ; \quad [M]_D^{25} = +4.78^\circ \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{+0.26^\circ \times 100}{1 \times 10.0} = +2.6^\circ; \quad [M]_D^{25} = +3.0^\circ \text{ (in absolute alcohol)}$$

2.985 mg. substance: 7.970 mg. CO_2 and 3.910 mg. H_2O
$C_7H_{17}N$. Calculated. C 72.95, H 14.88
115.1 Found. " 72.81, " 14.66

Dextro-Benzoyl-3-Aminoheptane—3-Aminoheptane hydrochloride, $[\alpha]_D^{25} = +1.00^\circ$ (10 per cent HCl), was converted into its benzoyl derivative. M.p. 66–68°.

$$[\alpha]_D^{25} = \frac{+0.20^\circ \times 100}{1 \times 10.0} = +2.0^\circ; \quad [M]_D^{25} = +4.4^\circ \text{ (in absolute alcohol)}$$

4.110 mg. substance: 11.510 mg. CO_2 and 3.398 mg. H_2O
$C_{14}H_{21}ON$. Calculated. C 76.65, H 9.66
219.2 Found. " 76.37, " 9.25

Dextro-3-Nonanol[8]—3-Nonanol was prepared from ethyl magnesium bromide and heptaldehyde. The carbinol was resolved by recrystallizing the cinchonidine salt of the phthalate from acetone. The less soluble salt gave a carbinol which boiled at 96–98°, $p = 19$ mm.

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[8] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, **103**, 1935 (1913).

$$[\alpha]_D^{25} = \frac{+5.83°}{1 \times 0.823} = +7.08°; \quad [M]_D^{25} = +10.2° \text{ (homogeneous)}$$

3.498 mg. substance: 9.595 mg. CO_2 and 4.380 mg. H_2O
$C_9H_{20}O$. Calculated. C 74.92, H 13.98
144.2 Found. " 74.80, " 14.01

Levo-3-Iodononane—30 gm. of 3-nonanol, $[\alpha]_D^{25} = +7.08°$ (homogeneous), were treated with anhydrous hydrogen iodide. The two sealed bomb tubes were allowed to stand overnight at room temperature. The iodide distilled at 99–100°, $p = 10$ mm. Yield 40 gm.

$$[\alpha]_D^{25} = \frac{-18.2°}{1 \times 1.28} = -14.2°; \quad [M]_D^{25} = -36.1° \text{ (homogeneous)}$$

4.235 mg. substance: 6.600 mg. CO_2 and 2.905 mg. H_2O
$C_9H_{19}I$. Calculated. C 42.51, H 7.54
254.1 Found. " 42.52, " 7.67

Dextro-3-Azidononane—40 gm. of 3-iodononane, $[\alpha]_D^{25} = -14.2°$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 40 cc. of water and 650 cc. of methanol. This was sealed into seven bomb tubes and heated for 10 hours at 100°. The azide was isolated as previously described. B.p. 105–107°, $p = 30$ mm. Yield 17 gm.; $d_4^{25} = 0.8575$ (*in vacuo*); $n_D^{25} = 1.4373$.

$$[\alpha]_{5780}^{25} = \frac{+5.22°}{2 \times 0.858} = +3.04°; \quad [M]_{5780}^{25} = +5.14° \text{ (homogeneous)}$$

4.598 mg. substance: 10.800 mg. CO_2 and 4.600 mg. H_2O
$C_9H_{19}N_3$. Calculated. C 63.84, H 11.32
169.2 Found. " 64.05, " 11.19

Dextro-3-Aminononane—15 gm. of 3-azidononane, $[\alpha]_{5780}^{25} = +3.04°$ (homogeneous), were dissolved in 40 cc. of methanol and 0.8 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 6 hours. The amine hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{+0.09° \times 100}{1 \times 6.0} = +1.5°; \quad [M]_D^{25} = +2.7° \text{ (in water)}$$

The free base was isolated and distilled from sodium. B.p. 102°, $p = 50$ mm. $d_4^{27} = 0.781$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+3.60}{1 \times 0.781} = +4.61°; \quad [M]_D^{27} = +6.60° \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{+0.37° \times 100}{1 \times 10.0} = +3.7°; \quad [M]_D^{25} = +5.3° \text{ (in absolute alcohol)}$$

3.060 mg. substance: 8.475 mg. CO_2 and 4.010 mg. H_2O

$C_9H_{21}N$.	Calculated.	C 75.43, H 14.79
143.2	Found.	" 75.52, " 14.66

Dextro-Benzoyl-3-Aminononane—3-Aminononane hydrochloride, $[\alpha]_D^{25} = +1.50°$ (in water), was converted into the benzoyl derivative. M.p. 86°.

$$[\alpha]_D^{25} = \frac{+1.25° \times 100}{1 \times 10.0} = +12.5°; \quad [M]_D^{25} = +30.9° \text{ (in absolute alcohol)}$$

4.690 mg. substance: 13.422 mg. CO_2 and 4.200 mg. H_2O

$C_{16}H_{25}ON$.	Calculated.	C 77.67, H 10.19
247.2	Found.	" 78.04, " 10.00

Levo-4-Octanol[9]—4-Octanol was resolved by recrystallizing the strychnine salt of the phthalate from 90 per cent acetone. The less soluble portion gave a carbinol which boiled at 79–80°, $p = 17$ mm.

$$[\alpha]_D^{25} = \frac{+0.52°}{1 \times 0.81} = +0.64°; \quad [M]_D^{25} = +0.83° \text{ (homogeneous)}$$

3.920 mg. substance: 10.61 mg. CO_2 and 4.860 mg. H_2O

$C_8H_{18}O$.	Calculated.	C 73.77, H 13.94
130.1	Found.	" 73.80, " 13.87

Levo-4-Iodooctane—20 gm. of 4-octanol, $[\alpha]_D^{25} = +0.64°$ (homogeneous), were treated with anhydrous hydrogen iodide as previously described. The iodide boiled at 97°, $p = 22$ mm. Yield 30 gm.; $d_4^{25} = 1.3233$ (*in vacuo*); $n_D^{25} = 1.4891$.

$$[\alpha]_D^{25} = \frac{-2.32°}{1 \times 1.32} = -1.76°; \quad [M]_D^{25} = -4.23° \text{ (homogeneous)}$$

5.158 mg. substance: 7.595 mg. CO_2 and 3.320 mg. H_2O

$C_8H_{17}I$.	Calculated.	C 39.99, H 7.14
240.1	Found.	" 40.15, " 7.20

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[9] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931).

Levo-4-Azidooctane—30 gm. of 4-iodooctane, $[\alpha]_D^{25} = -1.76°$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 30 cc. of water and 400 cc. of methanol. This was sealed into four bomb tubes and heated for 16 hours at 80°. The azide was isolated as usual. B.p. 92–93°, $p = 35$ mm. Yield 15 gm.; $d_4^{25} = 0.8572$ (*in vacuo*); $n_D^{25} = 1.4342$.

$$[\alpha]_D^{25} = \frac{-0.70°}{1 \times 0.857} = -0.82°; \quad [M]_D^{25} = -1.27° \text{ (homogeneous)}$$

4.675 mg. substance: 10.630 mg. CO_2 and 4.600 mg. H_2O

$C_8H_{17}N_3$.	Calculated.	C 61.87, H 11.05
155.2	Found.	" 62.06, " 11.01

Levo-4-Aminooctane—15 gm. of 4-azidooctane, $[\alpha]_D^{25} = -0.82°$ (homogeneous), were dissolved in 50 cc. of methanol and 1 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 6 hours. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{-0.10° \times 100}{1 \times 20.0} = -0.50°; \quad [M]_D^{25} = -0.83° \text{ (10\% HCl)}$$

3.422 mg. substance: 7.255 mg. CO_2 and 3.730 mg. H_2O

$C_8H_{20}NCl$.	Calculated.	C 57.96, H 12.18
165.6	Found.	" 57.81, " 12.19

The free base was liberated as usual. B.p. 92–93°, $p = 80$ mm. $d_4^{27} = 0.777$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+0.35°}{1 \times 0.78} = +0.45°; \quad [M]_D^{27} = +0.58° \text{ (homogeneous)}$$

4.922 mg. substance: 13.410 mg. CO_2 and 6.440 mg. H_2O

$C_8H_{19}N$.	Calculated.	C 74.33, H 14.83
129.2	Found.	" 74.29, " 14.64

Dextro-4-Aminooctane Benzoate—2 gm. of 4-aminooctane hydrochloride, $[\alpha]_D^{25} = -0.50°$ (10 per cent HCl), were converted into the benzoyl derivative. M.p. 99–100°.

$$[\alpha]_D^{25} = \frac{+0.26° \times 100}{1 \times 20.0} = +1.30°; \quad [M]_D^{25} = +3.03° \text{ (in absolute alcohol)}$$

3.570 mg. substance: 10.110 mg. CO_2 and 3.180 mg. H_2O

$C_{15}H_{23}ON$.	Calculated.	C 77.19, H 9.94
233.2	Found.	" 77.22, " 9.97

THE MECHANISM OF THE REACTION OF SUBSTITUTION AND WALDEN INVERSION

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It was emphasized in previous publications[1] that rigorous proof of the configurational relationship of secondary carbinols and corresponding halides and of hydroxy acids and corresponding halogeno acids had not yet been presented. On the other hand, it was established in our laboratory, on sufficient evidence, that amines and corresponding carbinols rotate in the same direction, and also that configurationally related secondary halides are of the same sign. Furthermore, Polanyi and Bergmann[2] as well as Hughes, Juliusburger, Masterman, Topley, and Weiss[3] reached the conclusion on good grounds that substitution of halogen for halogen is connected with an inversion of configuration.

It is now intended to use this information in an attempt to throw light on the configurational relationship of corresponding hydroxy and halogeno derivatives.

The argument on which this investigation is based is the following: If two consecutive reactions of substitution on the asymmetric carbon atom follow the same mechanism, either with or without inversion of configuration, then the final substance should have the configuration of the starting material.

In Table I are given a set of reactions through which it was attempted to obtain the desired information.

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[1] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **107**, 533 (1934). Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Physic.*, **4**, 442 (1936).

[2] Bergmann, E., Polanyi, M., and Szabo, A. L., *Tr. Faraday Soc.*, **32**, 843 (1936). von Hartel, H., Meer, N., and Polanyi, M., *Z. physik. Chem.*, *Abt. B*, **19**, 139 (1932).

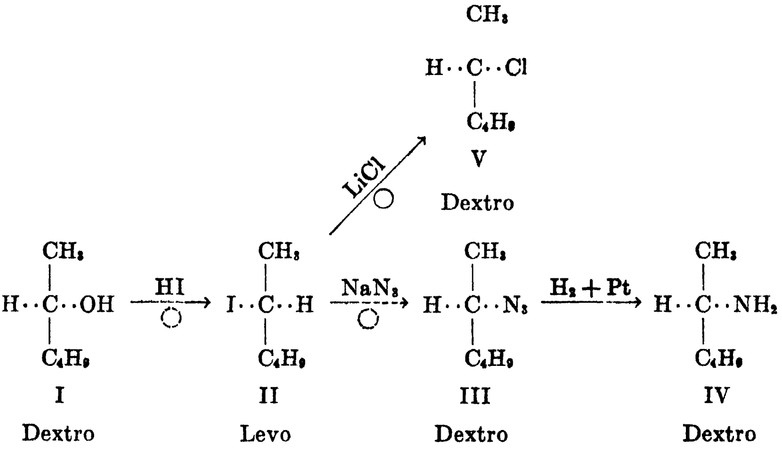
[3] Hughes, E. D., Juliusburger, F., Masterman, S., Topley, B., and Weiss, J., *J. Chem. Soc.*, 1525 (1935).

From Table I it can be seen that two consecutive reactions of substitution lead from a dextro-carbinol (I) to a dextro-amine (IV). It is evident that the amine (IV) is configurationally related to the azide (III); hence the azide (III) likewise is configurationally related to carbinol (I).

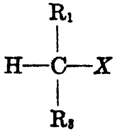
Unfortunately, there exists no definite information regarding the mechanism of either one of the two reactions of substitution. However, substitution of chlorine for iodine is connected with an inversion of configuration. True, doubt was expressed as to the identity of direction of rotation in two configurationally related secondary halides. This scepticism, however, is not warranted for the following reasons.

TABLE I

Reactions of Substitution in Saturated Derivatives



First, a series of substances of Type I



$$H-\overset{R_1}{\underset{R_2}{C}}-X$$

I

(where X = F, Cl, Br, I
R_1 and R_2 = normal alkyl groups)

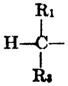
may in a sense be regarded as a homologous series, since the 4 halogen atoms are similar in their chemical properties and in the system of their absorption bands, but differ principally in their volume. Hence, if two members of this series differed in the direction of their rotation, the character of their rotatory dispersion should also be different. The latter, however, is not the case.

Second, as can be seen from Table II, levorotatory bromides and iodides all lead on the action of lithium chloride to dextrorotatory chlorides.

Hence, bromides and iodides rotating in the same direction are configurationally related. Thus it may be accepted that in the reactions given in Table I, substitution of the chlorine for the iodine atom is connected with an inversion of configuration. It may be mentioned that this is the first experimental evidence to the effect that substitution of a chlorine for another halogen is connected with an inversion of configuration.

TABLE II

Direction of Partial Rotations of Functional Groups OH, —Br, —I, or —Cl

 $H-\overset{R_1}{\underset{R_3}{C}}-$		-OH→	- Br or —I	$\xrightarrow{(LiCl)}$ —Cl
R_1	R_3			
n-Butyl	Vinyl	+	(Br) −	+
Ethyl	*n*-Hexyl	+	(I) −	+
n-Propyl	*n*-Butyl	+	“ −	+
Ethyl	Phenyl	+	(Br) −	+
n-Propyl	“	+ or −	“ −	+

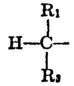
This reaction may serve as a key to explain the other reactions given in Table I. The azido group may be regarded as a pseudohalogen. It then seems legitimate to assume that the substitution of an azido group for a halogen atom proceeds similarly to that of 1 halogen atom for another. Therefore, the azido group, like a halogen atom, should approach the dipole from the positive end, and thus substitution should bring about an inversion of configuration. This assumption leads to the conclusion that the substitution of a halogen atom for the hydroxyl group in substances of Table I likewise is connected with an inversion of configuration. It may be seen from Table III that the set of reactions given in Table I has been observed in more than one case.

In their latest publication, Polanyi and Bergmann assumed that substitution of halogen for halogen is connected with inversion of configuration. It seems justified now to generalize this formulation for substances of Type I (X = a negative functional group) to the effect that substitution of any negative group or atom for another is connected with an inversion of configuration.

α-Substituted Carboxylic Acids—In α-substituted carboxylic acids a choice between the two possible mechanisms of the reaction of substitution is more difficult than in substances of Type I for the reason that ionization, lactone formation, enolization, etc., furnish frequently disturbing concomitant reactions; in addition, in the α-bromo-, or α-chlorocarboxylic acids, it is difficult to distinguish the partial rotations of the halogen atom from those of the carboxyl group. However, from the knowledge of configurational relationship of substances of Type I and of corresponding α-carboxylic acids, it is possible to correlate the mechanism of the reaction of substitution of these two groups of substances.

TABLE III

Direction of Rotations of Functional Groups OH, Hl, N_3, NH_2

 $\mathrm{H{-}}\overset{R_1}{\underset{R_2}{C}}{-}$		—OH →	—Hl →	—N_3 →	—NH_2
R_1	R_2				
n-Butyl	Vinyl	+	−	+	
Methyl	Ethyl	+	−	+	+
"	n-Butyl	+	−	+	+
"	n-Hexyl	+	−	+	+
Ethyl	n-Butyl	+	−	+	+
"	n-Hexyl	+	−	+	+
Propyl	n-Butyl	+	−	−	+
"	n-Amyl	+	−	−	+

In Table IV are given the configurational relationships of substances of Type I and of the corresponding α-substituted acids. The results of corresponding reactions of substitution are given in Table V.

From Table V it appears that substitution of a halogen atom for a hydroxyl group or of an amino group for a halogen atom

TABLE IV

Configurational Relationship of Substances $\begin{array}{c} R_1 \\ | \\ H\cdot\cdot C\cdot\cdot X \\ | \\ R_3 \end{array}$ *and* $\begin{array}{c} COOH \\ | \\ H\cdot\cdot C\cdot\cdot X \\ | \\ R_3 \end{array}$

(X = OH, Hl, NH_2; $R_1 < R_3$)*

$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot OH \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot OH \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot Hl \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot Hl \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot N_3 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot N_3 \\ \vert \\ R_3 \end{array}$
Dextro	Levo	Dextro	Levo	Dextro	Dextro	Dextro	Dextro

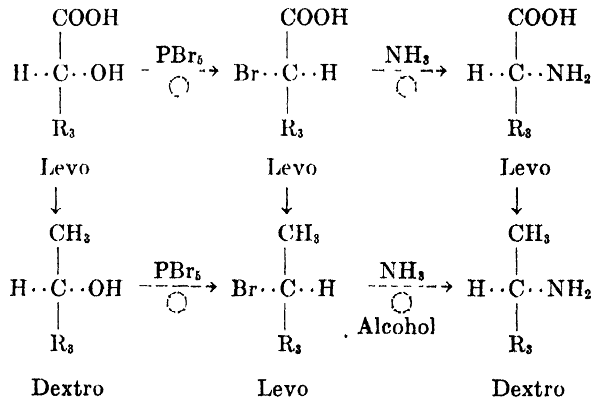
* The rotations of these substances are levo if $R_1 > R_3$.

proceeds similarly in the acids and in the alkyl derivatives. Furthermore, the fact that in the bromo and iodo derivatives the partial rotation of the halogen atom has the same sign in the acid and in the alkyl derivative permits the conclusion that the same relationship exists in the chloro derivatives. This is an important conclusion, because in the chloro acids the rotatory dispersion curve does not permit a definite decision as to which of the two polar groups furnishes the first partial rotation.

Vinyl Derivatives.—In this series of unsaturated compounds two consecutive reactions of substitution bring about an inversion

TABLE V

Reactions of Substitution

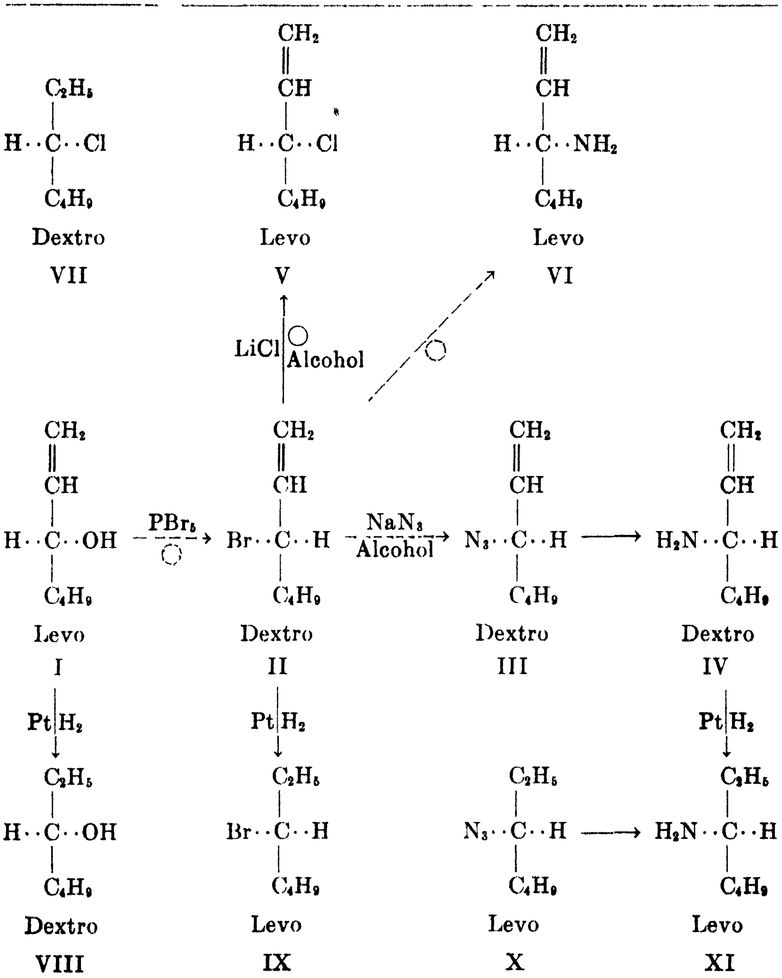


$$\begin{array}{ccccc}
\overset{\displaystyle \mathrm{COOH}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{H\cdot\cdot C\cdot\cdot OH}}} & \xrightarrow[\circlearrowleft]{\mathrm{PBr_5}} & \overset{\displaystyle \mathrm{COOH}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{Br\cdot\cdot C\cdot\cdot H}}} & \xrightarrow[\circlearrowleft]{\mathrm{NH_3}} & \overset{\displaystyle \mathrm{COOH}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{H\cdot\cdot C\cdot\cdot NH_2}}} \\
\text{Levo} & & \text{Levo} & & \text{Levo} \\
\downarrow & & \downarrow & & \downarrow \\
\overset{\displaystyle \mathrm{CH_3}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{H\cdot\cdot C\cdot\cdot OH}}} & \xrightarrow[\circlearrowleft]{\mathrm{PBr_5}} & \overset{\displaystyle \mathrm{CH_3}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{Br\cdot\cdot C\cdot\cdot H}}} & \xrightarrow[\substack{\circlearrowleft \\ \text{Alcohol}}]{\mathrm{NH_3}} & \overset{\displaystyle \mathrm{CH_3}}{\underset{\displaystyle \mathrm{R_3}}{\mathrm{H\cdot\cdot C\cdot\cdot NH_2}}} \\
\text{Dextro} & & \text{Levo} & & \text{Dextro}
\end{array}$$

of configuration (see second row of Table VI). This conclusion is warranted, since the configurationally related saturated derivatives may be substituted for each vinyl derivative (see third row of Table VI). Substitution of a halogen atom for a hydroxyl group, of 1 halogen atom for another, or of an amino group for a halogen atom proceeds similarly in the two series of unsaturated and saturated compounds. Only the substitution of an azido group for a halogen atom is different in these two series. Thus it may be assumed that this last reaction of substitution proceeds without change of configuration in the vinyl derivatives. It is also evident that the molecular structure and not the external conditions are responsible for the difference in the outcome of this reaction, inasmuch as the conditions of the reaction were similar in both series.

TABLE VI

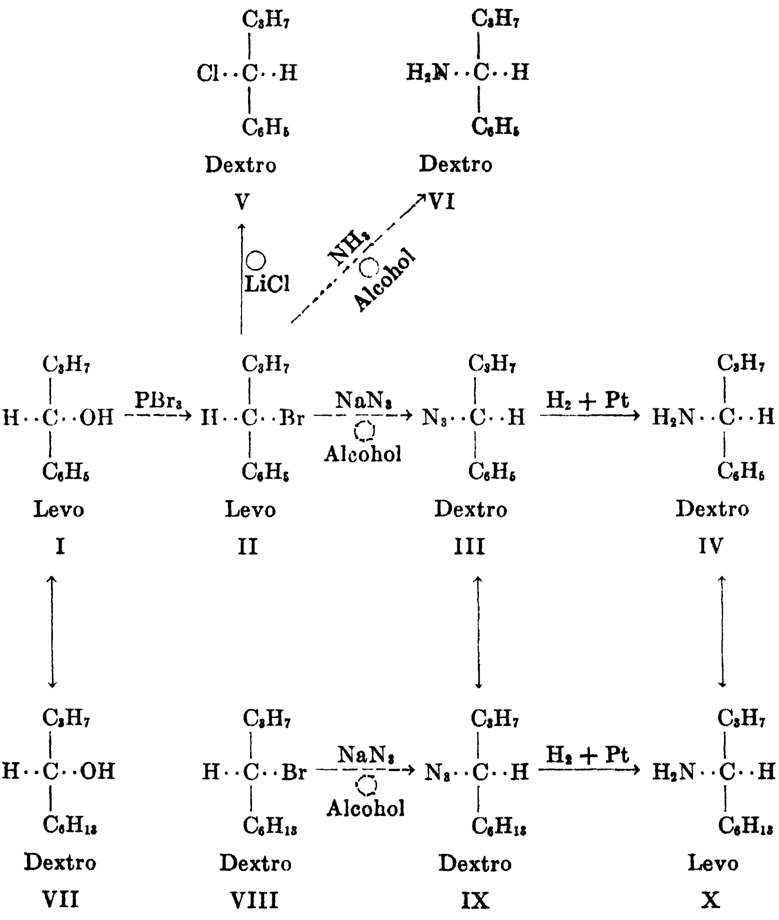
Reactions of Substitution in Unsaturated Derivatives



Alkylaryl Derivatives—In the group of substances given in Table VII two consecutive substitutions lead also to a change in configuration (see the second row), since the corresponding configurationally related alkyl derivatives can be substituted for all the alkylphenyl derivatives, except the halides. Furthermore, substitution of (1) 1 halogen atom for another, (2) an amino group for a halogen atom, (3) an azido group for a halogen atom proceeds with change of sign as in the alkyl series. In other words, the reaction of substi-

TABLE VII

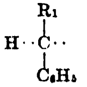
Reactions of Substitutions in Phenyl Derivatives


C_3H_7 / Cl··C··H / C_6H_5
Dextro
V
C_3H_7 / H_2N··C··H / C_6H_5
Dextro
VI
LiCl
NH_3
Alcohol
C_3H_7 / H··C··OH / C_6H_5
Levo
I
PBr_3
C_3H_7 / H··C··Br / C_6H_5
Levo
II
NaN_3
Alcohol
C_3H_7 / N_3··C··H / C_6H_5
Dextro
III
$H_2 + Pt$
C_3H_7 / H_2N··C··H / C_6H_5
Dextro
IV
C_3H_7 / H··C··OH / C_6H_{13}
Dextro
VII
C_3H_7 / H··C··Br / C_6H_{13}
Dextro
VIII
NaN_3
Alcohol
C_3H_7 / N_3··C··H / C_6H_{13}
Dextro
IX
$H_2 + Pt$
C_3H_7 / H_2N··C··H / C_6H_{13}
Levo
X

tution from (I) to (II) leads to substances correlated as are (VII) to (VIII); that is, in substance (I) the substitution of a halogen atom for a hydroxyl group proceeds without change of configuration. It must be mentioned that, if methyl or ethylphenyl carbinol is taken as the starting material, then the reactions of substitution follow a course analogous to that of the corresponding reaction in the alkyl series, as can be seen from Table VIII. Again it must be emphasized that the chloro derivatives of the ethylphenyl and of the propylphenyl carbinols are similar in their rotatory dispersion curves and hence are configurationally related, when they have the same sign of rotation.

TABLE VIII

Direction of Rotation of Derivatives Containing a Phenyl Group, the Bromides Being Prepared by the Action of HBr

 $H\cdot\cdot\overset{R_1}{\underset{C_6H_5}{C}}\cdot\cdot$	· OH	··Br	··N_3	··NH_2
R_1				
Methyl	+	−	+	+
Ethyl	+	−	+	+
Propyl	+	+	−	−

SUMMARY

In summing up the results recorded in Tables I, VI, and VII it may be stated that:

1. In normal saturated aliphatic derivatives, substitution on the asymmetric carbon by a negative group or atom is connected with an inversion of configuration.

2. In substances of Type I, having R_1 = —CH═CH_2, substitution of an azido group for a halogen proceeds without inversion of configuration.

3. In substances of Type I, having R_3 = —C_6H_5, substitution of Br for a hydroxyl by HBr or PBr_3 in the absence of pyridine proceeds without inversion of configuration, in the presence of pyridine with inversion of configuration.

4. A formulation of the mechanism of the reaction of substitution on the asymmetric carbon atom is possible at this time for a

limited group of substances, namely for the normal saturated alkyl derivatives.

5. For this group of substances, the reaction of substitution is connected with an inversion of configuration, provided the mechanism of substitution of an N_3 group for halogen proceeds by the same mechanism as the substitution of one halogen for another.

6. A general formulation of the mechanism of the reaction of substitution is not yet possible. Consequently, a general theory of Walden inversion likewise is not yet possible.

EXPERIMENTAL

Dextro-4-Chlorooctane—10 gm. of 4-iodooctane, $\alpha_D^{25} = -2.00°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 135 cc. of methanol. This was placed in an oven at 37° for 1 week. The reaction mixture was poured into a concentrated calcium chloride solution and the halide was extracted with pentane. The extract was dried with phosphoric anhydride, and the chloride was distilled. B.p. 92°, $p = 50$ mm. Yield 2 gm. $\alpha_D^{25} = +0.28°$ (homogeneous, 1 dm.).

3.516 mg. substance: 8.373 mg. CO_2 and 3.715 mg. H_2O
$C_8H_{17}Cl$. Calculated. C 64.60, H 11.53
148.6 Found. " 64.94, " 11.82

Dextro-3-Chlorononane—10 gm. of 3-iodononane, $\alpha_D^{25} = -18.2°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 180 cc. of methanol. This was placed in an oven at 37° for 6 days. (The reaction at room temperature was slow. After 3 days the iodide was recovered and its rotation was $-16.2°$.) The halide was isolated as usual. B.p. 98°, $p = 33$ mm. Yield 3 gm. $\alpha_D^{25} = +5.10°$ (homogeneous, 1 dm.).

4.200 mg. substance: 10.210 mg. CO_2 and 4.410 mg. H_2O
$C_9H_{19}Cl$. Calculated. C 66.42, H 11.78
162.6 Found. " 66.34, " 11.75

Levo-1-Hepten-3-ol (*Butylvinylcarbinol*)—The inactive carbinol was prepared and resolved as previously described.[4] The acid phthalic ester was, however, made by a slightly different procedure. The mixture of phthalic anhydride, pyridine, and carbinol was heated in a water bath kept at 75° for 3 hours. The subsequent procedure was unchanged. The insoluble strychnine salt, after recrystallization, yielded a carbinol which distilled at 103–106°, $p = 147$ mm.

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[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

$$[\alpha]_D^{25} = \frac{-19.30°}{1 \times 0.833} = -23.2°; \quad [M]_D^{25} = -26.5° \text{ (homogeneous)}$$

4.012 mg. substance: 10.835 mg. CO_2 and 4.440 mg. H_2O
$C_7H_{14}O$. Calculated. C 73.61, H 12.37
114.1 Found. " 73.64, " 12.38

Dextro-3-Chloro-1-Heptene—50 gm. of 1-hepten-3-ol, $[\alpha]_D^{25} = -23.2°$ (homogeneous), were treated with 110 gm. of phosphorus pentachloride in dry ether according to the procedure previously described.[5] The chloride distilled at 92–94°, $p = 125$ mm. Yield 44 gm.; $d_4^{25} = 0.8857$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+8.65°}{1 \times 0.886} = +9.76°; \quad [M]_D^{25} = +12.9° \text{ (homogeneous)}$$

3.410 mg. substance: 7.890 mg. CO_2 and 2.980 mg. H_2O
$C_7H_{13}Cl$. Calculated. C 63.37, H 9.88
132.6 Found. " 63.09, " 9.77

Levo-3-Azido-1-Heptene—44 gm. of 3-chloro-1-heptene, $[\alpha]_D^{25} = +9.76°$ (homogeneous), were added to a solution of 27 gm. of sodium azide (Kahlbaum) in 75 cc. of water and 530 cc. of methanol. The resulting solution was transferred into pressure bottles, and allowed to stand at room temperature (25°) for 1 week. An equal volume of concentrated calcium chloride solution was then added, and the azide was extracted with pentane. The extract was washed with concentrated calcium chloride solution and dried with anhydrous calcium sulfate. After removal of the solvent, the residue distilled at 78–81°, $p = 32$ mm. Yield 36 gm.; $d_4^{27} = 0.888$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{-0.14°}{4 \times 0.89} = -0.04°; \quad [M]_D^{27} = -0.06° \text{ (homogeneous)}$$

3.275 mg. substance: 7.225 mg. CO_2 and 2.810 mg. H_2O
$C_7H_{13}N_3$. Calculated. C 60.37, H 9.42
139.1 Found. " 60.16, " 9.60

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[5] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

Levo-3-Amino-1-Heptene—140 gm. of 3-chloro-1-heptene, $[\alpha]_D^{25} = +8.86°$ (homogeneous), were treated with ammonia in the following manner. 5 gm. of the halide were dissolved in 40 cc. of a 40 per cent solution of ammonia in methanol (which was made by adding liquid ammonia to cooled methanol) and sealed in a bomb tube. This was allowed to stand at 25° for 1 day, and was then heated at 50° for 3 days. The tube was cooled, opened, and the solution poured into ice. Concentrated sodium hydroxide solution was added, and the amine was extracted with ether. The extract was washed with concentrated sodium hydroxide solution, and dried with solid sodium hydroxide. The ether and methanol were distilled off at atmospheric pressure. The primary amine distilled at 95–105°, $p = 155$ mm. Yield 31 gm. $\alpha_D^{25} = -4.80°$ (homogeneous, 1 dm.).

3.289 mg. substance: 8.910 mg. CO_2 and 3.950 mg. H_2O
$C_7H_{15}N$. Calculated. C 74.25, H 13.36
113.1 Found. " 73.87, " 13.43

The higher fractions contained secondary and tertiary amines. A pure secondary amine was obtained from a chloride of $[\alpha]_D^{25} = -6.2°$ (homogeneous). B.p. 92–95°, $p = 1$ mm. $\alpha_D^{25} = +0.52°$ (homogeneous, 1 dm.).

3.002 mg. substance: 8.880 mg. CO_2 and 3.514 mg. H_2O
$C_{14}H_{27}N$. Calculated. C 80.29, H 13.01
209.24 Found. " 80.66, " 13.10

Levo-3-Aminoheptane—30 gm. of 3-azido-1-heptene, $[\alpha]_D^{27} = -0.04°$ (homogeneous), were dissolved in 70 cc. of methanol, and 1.4 gm. of Adams' catalyst were added. This was shaken (in two portions) overnight with hydrogen at a pressure of 3 atmospheres. The catalyst was then filtered off, and hydrogen chloride in methanol was added to the filtrate. This was evaporated to dryness under reduced pressure. The crystals were taken up in 50 per cent potassium hydroxide and extracted with ether. The extract was dried over metallic sodium. The amine distilled at 100–106°, $p = 148$ mm.

$$[\alpha]_D^{27} = \frac{-0.016°}{1 \times 0.783} = -0.02°; \qquad [M]_D^{27} = -0.02° \text{ (homogeneous)}$$

4.221 mg. substance: 11.340 mg. CO_2 and 5.525 mg. H_2O
$C_7H_{17}N$. Calculated. C 72.95, H 14.88
115.1 Found. " 73.26, " 14.64

Levo-3-Aminoheptane—10 gm. of 3-amino-1-heptene, α_D^{25} = +0.8° (homogeneous, 1 dm.), were dissolved in 25 cc. of methanol and 0.5 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres overnight. The free amine was isolated as described above. B.p. 99–101°, p = 150 mm.

$$[\alpha]_D^{27} = \frac{-0.35^\circ}{1 \times 0.783} = -0.45^\circ; \quad [M]_D^{27} = -0.52^\circ \text{ (homogeneous)}$$

4.159 mg. substance: 11.090 mg. CO_2 and 5.490 mg. H_2O

$C_7H_{17}N$. Calculated. C 72.95, H 14.88
115.1 Found. " 72.71, " 14.77

Levo-3-Bromo-1-Heptene—20 gm. of 1-hepten-3-ol, $[\alpha]_D^{25}$ = +10.5° (homogeneous), were dissolved in 30 cc. of dry ether, and added dropwise into a cooled suspension of 105 gm. of phosphorus pentabromide in 200 cc. of dry ether, with stirring. After the carbinol was added, the solution was allowed to stand for 1 hour at room temperature. The bromide was isolated as usual.[5] B.p. 92–94°, p = 50 mm. Yield 10 gm.; d_4^{25} = 1.164 (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-5.40^\circ}{1 \times 1.164} = -4.64^\circ; \quad [M]_D^{25} = -8.22^\circ \text{ (homogeneous)}$$

This rotation remained unchanged after 6 weeks standing at a temperature of about 5°.

3.214 mg. substance: 5.600 mg. CO_2 and 2.170 mg. H_2O

$C_7H_{13}Br$. Calculated. C 47.45, H 7.40
177.0 Found. " 47.51, " 7.55

Dextro-3-Chloro-1-Heptene—8.5 gm. of 3-bromo-1-heptene, α_D^{25} = −5.40° (homogeneous, 1 dm.), were added to a solution of 11 gm. of dry lithium chloride in 100 cc. of methanol (the warm solution of the salt was cooled before the bromide was added) and let stand at room temperature (about 25°) for 10 days. The solution was then poured into concentrated calcium chloride solution and extracted with pentane. The extract was washed with concentrated calcium chloride solution and dried with phosphoric anhydride overnight. After filtration and removal of the solvent, the residue was distilled. B.p. 87–88°, $p = 90$ mm. Yield 2 gm.

$$[\alpha]^{25}_{D} = \frac{+0.80°}{1 \times 0.89} = +0.90°; \qquad [M]^{25}_{D} = +1.19° \text{ (homogeneous)}$$

5.024 mg. substance: 11.700 mg. CO_2 and 4.410 mg. H_2O
5.012 " " : 5.425 " AgCl
$C_7H_{13}Cl$. Calculated. C 63.37, H 9.88, Cl 26.75
132.6 Found. " 63.50, " 9.82, " 26.77

Levo-1-Phenyl-1-Ethanol (*Methylphenylcarbinol*)[6]—The inactive carbinol was prepared by a Grignard reaction with bromobenzene and acetaldehyde. The carbinol was converted into the acid phthalic ester with pyridine. The phthalate was recrystallized from a mixture of ether and pentane. The pure phthalate melted at 108°. 1080 gm. of the phthalate were converted into the brucine salt in acetone and the salt recrystallized from 95 per cent acetone fifteen times. The phthalate, which was recovered from the original mother liquor, partially crystallized on standing. The crystals were filtered and on hydrolysis gave 16 gm. of a carbinol with a rotation of $\alpha^{25}_{D} = +1.3°$ (homogeneous, 1 dm.). The filtrate gave 20 gm. of a carbinol with a rotation of $\alpha^{25}_{D} = +30°$. Mother Liquors 2 to 8 were combined and the phthalate recovered. The crystalline phthalate gave 58 gm. of carbinol, $\alpha^{25}_{D} = +2.3°$, and the filtrate yielded 62 gm. of carbinol, $\alpha^{25}_{D} = +37°$. Mother Liquors 8 to 12 were treated the same way. The crystalline phthalate gave 54 gm. of carbinol, $\alpha^{25}_{D} = -0.5°$, and the filtrate yielded 17 gm. of carbinol, $\alpha^{25}_{D} = -30°$.

The insoluble brucine salt yielded 35 gm. of carbinol. B.p. 75°, $p = 1$ mm.

$$[\alpha]^{25}_{D} = \frac{-42.0°}{1 \times 1.00} = -42.0°; \qquad [M]^{25}_{D} = -51.3° \text{ (homogeneous)}$$

4.377 mg. substance: 12.585 mg. CO_2 and 3.210 mg. H_2O
$C_8H_{10}O$. Calculated. C 78.64, H 8.26
122.1 Found. " 78.40, " 8.20

Levo-1-Chloro-1-Phenylethane (*Methylphenylchloromethane*)[7]—38 gm. of 1-phenyl-1-ethanol, $[\alpha]^{25}_{D} = -36.3°$ (homogeneous), were dissolved in 50 gm. of redistilled thionyl chloride with cooling. The solution was allowed to stand at room temperature for 10 minutes and then was refluxed for 15 minutes. The reaction mixture was poured into ice and the chloride was extracted with pentane. The extract was washed with water and dried with phosphoric anhydride. The chloride distilled at 101°, $p = 50$ mm. Yield 39 gm.

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[6] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 355 (1926).

[7] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 71 (1911).

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-25.2^\circ}{1 \times 1.05} = -24.0^\circ; \qquad [M]_{\mathrm{D}}^{25} = -33.7^\circ \text{ (homogeneous)}$$

4.734 mg. substance: 11.900 mg. CO_2 and 2.790 mg. H_2O

C_8H_9Cl. Calculated. C 68.31, H 6.45
140.5 Found. " 68.54, " 6.59

Dextro-1-Azido-1-Phenylethane (*Methylphenylazidomethane*)—39 gm. of 1-chloro-1-phenylethane, $[\alpha]_{\mathrm{D}}^{25} = -24.0^\circ$ (homogeneous), were added to a solution of 27 gm. of sodium azide (Kahlbaum) in 70 cc. of water and 475 cc. of methanol. The solution was refluxed for 5 hours. The azide was isolated as usual. B.p. 114°, $p = 50$ mm. Yield 22 gm.; $d_4^{25} = 1.0321$ (*in vacuo*); $n_{\mathrm{D}}^{25} = 1.5233$.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+19.2^\circ}{1 \times 1.03} = +18.6^\circ; \qquad [M]_{\mathrm{D}}^{25} = +27.4^\circ \text{ (homogeneous)}$$

5.410 mg. substance: 13.005 mg. CO_2 and 3.100 mg. H_2O

$C_8H_9N_3$. Calculated. C 65.27, H 6.17
147.1 Found. " 65.55, " 6.41

Dextro-1-Amino-1-Phenylethane (*Methylphenylaminomethane*)—20 gm. of 1-azido-1-phenylethane, $[\alpha]_{\mathrm{D}}^{25} = +18.6^\circ$ (homogeneous), were dissolved in 50 cc. of methanol, 0.6 gm. of Adams' catalyst was added, and the mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride and the free base were isolated as usual. The amine boiled at 75°, $p = 15$ mm. Yield 5 gm.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+2.98^\circ}{1 \times 0.95} = +3.14^\circ; \qquad [M]_{\mathrm{D}}^{25} = +3.80^\circ \text{ (homogeneous)}$$

3.992 mg. substance: 11.605 mg. CO_2 and 3.220 mg. H_2O

$C_8H_{11}N$. Calculated. C 79.27, H 9.16
121.1 Found. " 79.27, " 9.03

Levo-1-Phenyl-1-Propanol (Ethylphenylcarbinol)[6]—The inactive carbinol was made from ethyl magnesium bromide and benzaldehyde. It was converted into the acid phthalic ester with pyridine. The phthalate was converted into the strychnine salt which was recrystallized from ethyl acetate four times. The crystals yielded 85 gm. of carbinol. B.p. 94–95°, $p = 10$ mm.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-22.0°}{1 \times 0.99} = -22.2°; \qquad [\mathrm{M}]_{\mathrm{D}}^{25} = -30.2° \text{ (homogeneous)}$$

3.024 mg. substance: 8.805 mg. CO_2 and 2.410 mg. CO_2
$C_9H_{12}O$. Calculated. C 79.35, H 8.89
136.1 Found. " 79.40, " 8.91

Levo-1-Chloro-1-Phenylpropane (Ethylphenylchloromethane)[6, 8]—75 gm. of 1-phenyl-1-propanol, $[\alpha]_{\mathrm{D}}^{25} = -22.2°$ (homogeneous), were cooled in an ice-alcohol bath, and 110 gm. of freshly distilled thionyl chloride were slowly added. The solution was allowed to stand at room temperature for 30 minutes and then was refluxed for 15 minutes. The chloride was isolated as usual. B.p. 77–80°, $p = 10$ mm. Yield 57 gm.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-29.8°}{1 \times 1.03} = -28.9°; \qquad [\mathrm{M}]_{\mathrm{D}}^{25} = -44.7° \text{ (homogeneous)}$$

3.514 mg. substance: 9.010 mg. CO_2 and 2.270 mg. H_2O
$C_9H_{11}Cl$. Calculated. C 69.88, H 7.18
154.6 Found. " 69.92, " 7.22

Levo-1-Bromo-1-Phenylpropane (Ethylphenylbromomethane)— 30 gm. of 1-phenyl-*l*-propanol, $[\alpha]_{\mathrm{D}}^{25} = +13.1°$ (homogeneous), were dissolved in 24 gm. of dry pyridine, and slowly added to a cooled suspension of 90 gm. of phosphorus pentabromide in 150 cc. of chloroform. The reaction mixture was then allowed to stand at 25° for 4 hours. The bromide was isolated as usual. B. p. 57–61°, $p = 0.6$ mm. Yield 24 gm.

$$\alpha_{\mathrm{D}}^{25} = -47.7° \text{ (homogeneous)}$$

5.994 mg. substance: 11.915 mg. CO_2 and 3.010 mg. H_2O
$C_9H_{11}Br$. Calculated. C 54.27, H 5.57
199.0 Found. " 54.20, " 5.61

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[8] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 69 (1911).

Dextro-1-Azido-1-Phenylpropane (*Ethylphenylazidomethane*)—57 gm. of 1-chloro-1-phenylpropane, $[\alpha]_D^{25} = -28.9°$ (homogeneous), were added to a solution of 40 gm. of sodium azide in 130 cc. of water and 730 cc. of methanol. The solution was refluxed for 16 hours. The azide was isolated as usual. B.p. 100–101°, $p = 22$ mm. Yield 30 gm. $\alpha_D^{25} = +32.95°$ (homogeneous, 1 dm.).

3.091 mg. substance: 7.604 mg. CO_2 and 1.930 mg. H_2O
$C_9H_{11}N_3$. Calculated. C 67.03, H 6.88
161.1 Found. " 67.08, " 6.98

Dextro-1-Amino-1-Phenylpropane (*Ethylphenylaminomethane*)—27 gm. of 1-azido-1-phenylpropane, $\alpha_D^{25} = +32.95°$ (homogeneous, 1 dm.), were dissolved in 70 cc. of methanol and 1.5 gm. of Adams' catalyst were added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride and the free base were isolated as usual. The amine boiled at 81°, $p = 10$ mm. Yield 14 gm.

$$[\alpha]_D^{25} = \frac{+4.25°}{1 \times 0.93} = +4.57°; \qquad [M]_D^{25} = +6.17° \text{ (homogeneous)}$$

4.604 mg. substance: 13.490 mg. CO_2 and 3.990 mg. H_2O
$C_9H_{13}N$. Calculated. C 79.93, H 9.70
135.1 Found. " 79.90, " 9.69

Dextro-1-Chloro-1-Phenylpropane—8 gm. of 1-bromo-1-phenylpropane, $\alpha_D^{25} = -8.04°$ (homogeneous, 1 dm.), prepared according to Levene and Mikeska,[6] were added to a cooled solution of 9 gm. of lithium chloride in 82 cc. of methanol. This was allowed to stand at room temperature for 3 days. The halide was isolated as described above. B.p. 87–89°, $p = 18$ mm. Yield 2 gm. $\alpha_D^{25} = +2.05°$ (homogeneous, 1 dm.).

4.002 mg. substance: 10.245 mg. CO_2 and 2.640 mg. H_2O
$C_9H_{11}Cl$. Calculated. C 69.88, H 7.18
154.6 Found. " 69.81, " 7.38

Dextro-1-Amino-1-Phenylpropane—24 gm. of 1-bromo-1-phenylpropane, $\alpha_D^{25} = -47.7°$ (homogeneous, 1 dm.), were dissolved in 300 cc. of a 40 per cent solution of ammonia in methanol (made by adding liquid ammonia to cooled methanol). The solution was placed in two magnesia bottles and allowed to stand at 25° for 4 days. The bottles were then cooled and opened. The solution was slowly dropped into 250 cc. of concentrated aqueous hydrochloric acid which was cooled in a freezing mixture. The amine was isolated as described for the 3-amino-1-heptene. B.p. 88–90°, p = 16 mm. Yield 6 gm.

$$[\alpha]_D^{25} = \frac{+3.39^\circ}{1 \times 0.93} = +3.65^\circ; \quad [M]_D^{25} = +4.93^\circ \text{ (homogeneous)}$$

3.878 mg. substance: 11.365 mg. CO_2 and 3.325 mg. H_2O
$C_9H_{13}N$. Calculated. C 79.93, H 9.70
135.1 Found. " 79.91, " 9.59

Levo-1-Phenyl-1-Butanol (Propylphenylcarbinol)[6,9]—The inactive carbinol was resolved by recrystallization of the strychnine salt of the acid phthalic ester. A carbinol was obtained from the crystals which boiled at 121–123°, p = 18 mm. The carbinol crystallized. The rotation of a melted portion was determined.

$$[\alpha]_D^{25} = \frac{-7.70^\circ}{1 \times 1.01} = -7.62^\circ; \quad [M]_D^{25} = -11.4^\circ \text{ (homogeneous)}$$

3.625 mg. substance: 10.625 mg. CO_2 and 3.090 mg. H_2O
$C_{10}H_{14}O$. Calculated. C 79.94, H 9.40
150.1 Found. " 79.92, " 9.53

Levo-1-Cyclohexyl-1-Butanol (Propylcyclohexylcarbinol)[9]—10 gm. of 1-phenyl-1-butanol, $[\alpha]_D^{25}$ = −7.62° (homogeneous), were dissolved in 25 cc. of a solution containing 90 gm. of methanol and 10 gm. of glacial acetic acid,[10] and 0.5 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 16 hours. The pressure change was 21 pounds (Burgess Parr Company, Illinois, model apparatus). The catalyst was filtered off, and the filtrate was distilled. The carbinol boiled at 76–77°, p = 1.5 mm. Yield 6.5 gm.

$$[\alpha]_D^{25} = \frac{-4.15^\circ}{1 \times 0.90} = -4.61^\circ; \quad [M]_D^{25} = -7.20^\circ \text{ (homogeneous)}$$

5.096 mg. substance: 14.390 mg. CO_2 and 5.815 mg. H_2O
$C_{10}H_{20}O$. Calculated. C 76.84, H 12.91
156.2 Found. " 77.00, " 12.77

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[9] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).

[10] Levene, P. A., *J. Biol. Chem.*, **115**, 275 (1936).

Dextro-1-Bromo-1-Phenylbutane (from Levo-1-Phenyl-1-Butanol)—27 gm. of 1-phenyl-1-butanol, $[\alpha]_D^{25} = -7.62°$ (homogeneous), were dissolved in 18 gm. of dry pyridine, and then slowly added to a cooled suspension of 75 gm. of phosphorus pentabromide (Kahlbaum) in 75 cc. of dry chloroform. The mixture was allowed to stand at room temperature with occasional shaking for 3 hours. (All of the phosphorus pentabromide did not react.) The bromide was isolated as usual. The pentane extract was dried over phosphoric anhydride. The bromide distilled at 67–75°, $p = 0.5$ to 1 mm. Yield 33 gm.; $d_4^{25} = 1.2631$ (*in vacuo*); $n_D^{25} = 1.5421$.

$$[\alpha]_D^{25} = \frac{+22.2°}{1 \times 1.26} = +17.6°; \qquad [M]_D^{25} = +37.5° \text{ (homogeneous)}$$

4.615 mg. substance: 9.515 mg. CO_2 and 2.500 mg. H_2O
$C_{10}H_{13}Br$. Calculated. C 56.33, H 6.15
213.0 Found. " 56.22, " 6.06

Levo-1-Bromo-1-Phenylbutane (from Levo-1-Phenyl-1-Butanol)[6]—8.5 gm. of 1-phenyl-1-butanol, $[\alpha]_4^{25} = -7.48°$ (homogeneous), were added to a cooled suspension of 25 gm. of phosphorus pentabromide (Kahlbaum) in 25 cc. of dry chloroform. The mixture was allowed to stand at room temperature for 3 hours. (All of the phosphorus pentabromide dissolved.) The bromide was isolated as usual. B.p. 67–72°, $p = 0.5$ to 1 mm. Yield 8 gm.

$$[\alpha]_D^{25} = \frac{-2.50°}{1 \times 1.26} = -1.98°; \qquad [M]_D^{25} = -4.22° \text{ (homogeneous)}$$

30 gm. of 1-phenyl-1-butanol, $[\alpha]_D^{25} = -7.48°$ (homogeneous), were cooled, and 60 gm. of phosphorus tribromide were slowly added. The solution was heated on the steam bath for 45 minutes. The bromide was isolated as usual. B.p. 65–72°, $p = 0.5$ to 1 mm. Yield 38 gm.

$$[\alpha]_D^{25} = \frac{-0.25°}{1 \times 1.26} = -0.20°; \qquad [M]_D^{25} = -0.42° \text{ (homogeneous)}$$

When phosphorus pentachloride was used with the pyridine solution under the same conditions, the chloride decomposed completely before distillation. A hydrocarbon which boiled at 147–152°, $p = 1.5$ mm., was obtained. Analysis showed the composition to be $C_{10}H_{12}$.

Levo-1-Chloro-1-Phenylbutane—7.5 gm. of 1-bromo-1-phenylbutane, $\alpha_D^{25} = +18.0°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 130 cc. of methanol. The resulting solution was allowed to stand at 50° for 36 hours. The halide was isolated as usual. A mixture of hydrocarbon, chloride, and unchanged bromide was obtained. The fraction corresponding to the chloride distilled at 119–128°, $p = 37$ mm., and had a rotation of $\alpha_D^{25} = -2.70°$ (homogeneous, 1 dm.).

4.204 mg. substance: 10.820 mg. CO_2 and 2.715 mg. H_2O
5.282 " " : 4.805 " AgCl

$C_{10}H_{13}Cl$. Calculated. C 71.19, H 7.77, Cl 21.04
168.6 Found. " 70.18, " 7.22, " 22.56

Levo-1-Azido-1-Phenylbutane (*Propylphenylazidomethane*)—12 gm. of 1-bromo-1-phenylbutane, $[\alpha]_D^{25} = +10.7°$ (homogeneous), were added to a solution of 12 gm. of sodium azide in 30 cc. of water and 200 cc. of methanol. The solution was allowed to stand for 16 hours at 25°. The azide was isolated as usual. B.p. 81–87°, $p = 3$ mm. Yield 9 gm.; $d_4^{25} = 0.9893$ (*in vacuo*); $n_D^{25} = 1.5122$.

$$[\alpha]_D^{25} = \frac{-9.01°}{1 \times 0.989} = -9.11°; \quad [M]_D^{25} = -16.0° \text{ (homogeneous)}$$

30 gm. of 1-bromo-1-phenylbutane, $[\alpha]_D^{25} = +17.6°$ (homogeneous), yielded 18 gm. of an azide which boiled at 85–90°, $p = 4$ mm.

$$[\alpha]_D^{25} = \frac{-15.2°}{1 \times 0.989} = -15.4°; \quad [M]_D^{25} = -27.0° \text{ (homogeneous)}$$

4.630 mg. substance: 11.695 mg. CO_2 and 3.155 mg. H_2O

$C_{10}H_{13}N_3$. Calculated. C 68.52, H 7.48
175.1 Found. " 68.88, " 7.62

Levo-1-Amino-1-Phenylbutane—27 gm. of 1-azido-1-phenylbutane, $[\alpha]_D^{25} = -13.2°$ (homogeneous), were dissolved in 50 cc. of methanol, and 1 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{2 \times 10.6} = +0.66°; \quad [M]_D^{25} = +1.22° \text{ (in water)}$$

The free amine was isolated and distilled at 105°, $p = 10$ mm. Yield 15 gm.; $d_4^{25} = 0.9197$ (*in vacuo*); $n_D^{25} = 1.5089$.

$$[\alpha]_D^{25} = \frac{-2.10^\circ}{1 \times 0.92} = -2.28^\circ; \qquad [M]_D^{25} = -3.40^\circ \text{ (homogeneous)}$$

3.802 mg. substance: 11.200 mg. CO_2 and 8.420 mg. H_2O

$C_{10}H_{15}N$. Calculated. C 80.47, H 10.14
149.1 Found. " 80.33, " 10.06

Levo-1-Amino-1-Phenylbutane—27 gm. of 1-bromo-1-phenylbutane, $\alpha_D^{25} = +8.80^\circ$ (homogeneous), were dissolved in 300 cc. of 40 per cent ammonia in methanol and treated exactly as described for the 1-amino-1-phenylpropane. The amine distilled at 103–104°, $p = 15$ mm. Yield 6 gm.

$$[\alpha]_D^{25} = \frac{-0.76^\circ}{1 \times 0.92} = -0.83^\circ; \qquad [M]_D^{25} = -1.24^\circ \text{ (homogeneous)}$$

3.929 mg. substance: 11.604 mg. CO_2 and 3.565 mg. H_2O

$C_{10}H_{15}N$. Calculated. C 80.47, H 10.14
149.1 Found. " 80.54, " 10.15

INDEX TO AUTHORS

A

Almquist, H. J. Further studies on the antihemorrhagic vitamin, 635

Alving, Alf S., and **Gordon, Wayne.** Studies of urea, creatinine, and ammonia excretion in dogs in acidosis, 103

Andrews, James C. See RUTENBER and ANDREWS, 203

Armstrong, W. D., and **Brekhus, P. J.** Chemical constitution of enamel and dentin. I. Principal components, 677

Arnow, L. Earle. The formation of dopa by the exposure of tyrosine solutions to ultraviolet radiation, 151

B

Barnard, Robert D. The reactions of nitrite with hemoglobin derivatives, 177

Behrens, Otto K., and **du Vigneaud, Vincent.** The synthesis of anserine from *l*-1-methylhistidine, 517

Bergman, A. J., and **Turner, C. W.** The composition of rabbit milk stimulated by the lactogenic hormone, 21

Block, Richard J. Chemical studies on the neuroproteins. II. The effect of age on the amino acid composition of human and mammalian brain proteins, 467

Blunden, Harry. See BUTTS, BLUNDEN, and DUNN, 289

Bodansky, Aaron. Notes on the determination of serum inorganic phosphate and serum phosphatase activity, 167

Bodansky, Oscar. The use of different measures of reaction velocity in the study of the kinetics of biochemical reactions, 555

Brekhus, P. J. See ARMSTRONG and BREKHUS, 677

Brodie, Bernard B., and **Friedman, Max M.** The determination of thiocyanate in tissues, 511

Browne, J. S. L. See VENNING, EVELYN, HARKNESS, and BROWNE, 225

Burk, Norval F. Osmotic pressure, molecular weight, and stability of amandin and excelsin and certain other proteins, 63

Butts, Joseph S., Blunden, Harry, and **Dunn, Max S.** Studies in amino acid metabolism. III. The fate of *dl*-leucine, *dl*-norleucine, and *dl*-isoleucine in the normal animal, 289

C

Campbell, Harold A., and Link, Karl Paul. Derivatives of *d*-galacturonic acid. III. The synthesis of a mercaptal of *d*-galacturonic acid and aldehydo tetraacetylmethyl-*d*-galacturonate, 471

Chaikoff, I. L. See Kaplan and Chaikoff, 647

Christman, Clarence C. See Levene and Christman, 575

Cori, Carl F., Cori, Gerty T., and Hegnauer, Albert H. Resynthesis of muscle glycogen from hexosemonophosphate, 193

Cori, Gerty T. See Cori, Cori, and Hegnauer, 193

Corley, Ralph C. See Leighty and Corley, 331

Craig, Lyman C. See Jacobs and Craig, 447

D

Deuel, Harry J., Jr., Murray, Sheila, Hallman, Lois F., and Tyler, David B. Studies on ketosis. XII. The effect of choline on the ketonuria of fasting rats following a high fat diet, 277

Dobriner, Konrad. Porphyrin excretion in the feces in normal and pathological conditions, 115

Dunn, Max S. See Butts, Blunden, and Dunn, 289

E

Evans, E. A., Jr. See Rittenberg, Schoenheimer, and Evans, 503

Evelyn, Kenneth A. See Venning, Evelyn, Harkness, and Browne, 225

F

Fenn, W. O., and Goettsch, Marianne. Electrolytes in nutritional muscular dystrophy in rabbits, 41

Friedman, Max M. See Brodie and Friedman, 511

G

Goettsch, Marianne. See Fenn and Goettsch, 41

Gordon, Wayne. See Alving and Gordon, 103

Gould, R. Gordon, Jr. See Jacobs and Gould, 141

Graham, W. R., Jr., Houchin, O. B., and Turner, C. W. The production of urea in the mammary gland, 29

H

Hallman, Lois F. See Deuel, Murray, Hallman, and Tyler, 277

Hamill, William H. See Stekol and Hamill, 531

Harger, R. N., Hulpieu, H. R., and Lamb, E. B. The speed with which various parts of the body reach equilibrium in the storage of ethyl alcohol, 689

Harkness, E. V. See Venning, Evelyn, Harkness, and Browne, 225

Harrer, Carter J. See Stotz, Harrer, Schultze, and King, 129

Harrison, Harold E. The sodium content of bone and other calcified material, 457

Hawkins, Nora C. See MORGAN, KIMMEL, and HAWKINS, 85

Hegnauer, Albert H. See CORI, CORI, and HEGNAUER, 193

Hess, W. C. See SULLIVAN and HESS, 537

Hoehn, Willard M. See MASON, HOEHN, McKENZIE, and KENDALL, 719

Hoffman, William S. The photoelectric determination of potassium in minute quantities of serum, 57

—. A rapid photoelectric method for the determination of glucose in blood and urine, 51

Hogden, Corinne G. See ROBINSON, PRICE, and HOGDEN, 481

Hogness, T. R., Sidwell, A. E., Jr., and Zscheile, F. P., Jr. The absorption spectra of compounds related to the sterols, 239

Houchin, O. B. See GRAHAM, HOUCHIN, and TURNER, 29

Hulpieu, H. R. See HARGER, HULPIEU, and LAMB, 689

J

Jacobs, Walter A., and Craig, Lyman C. The veratrine alkaloids. II. Further study of the basic degradation products of cevine, 447

— **and Gould, R. Gordon, Jr.** The ergot alkaloids. XII. The synthesis of substances related to lysergic acid, 141

Johnston, Charles G. See SCHOENHEIMER and JOHNSTON, 499

Jones, Chase Breese, and du Vigneaud, Vincent. The synthesis of hexocystine and hexomethionine and a study of their physiological availability, 11

K

Kaplan, A., and Chaikoff, I. L. The effect of choline on the lipid metabolism of blood and liver in the completely depancreatized dog maintained with insulin, 647

Kendall, Edward C. See MASON, HOEHN, McKENZIE, and KENDALL, 719

Kimmel, Louise. See MORGAN, KIMMEL, and HAWKINS, 85

King, C. G. See STOTZ, HARRER, SCHULTZE, and KING, 129

Kreider, Leonard C. See LEVENE and KREIDER, 591, 597

Kuna, Martin. See LEVENE, ROTHEN, and KUNA, 759, 777

L

Lamb, E. B. See HARGER, HULPIEU, and LAMB, 689

Lavietes, Paul H. Anaerobic ultrafiltration, 267

Leighty, John A., and Corley, Ralph C. Amino acid catabolism. IV. The fate of certain synthetic α-amino acids administered by subcutaneous injection to the normal dog, 331

Levene, P. A., and **Christman, Clarence C.** On a catalytically induced reaction resembling the Cannizzaro reaction, 575

— and **Kreider, Leonard C.** Oxidation and hydrolysis of polygalacturonide methyl ester to levo-tartaric acid, 591

— and —. The ring structure of α-methyl-*d*-galacturonide and its derivatives, 597

—, **Rothen, Alexandre**, and **Kuna, Martin.** The mechanism of the reaction of substitution and Walden inversion, 777

—, —, and —. Rotatory dispersion of configurationally related amines, 759

— and **Tipson, R. Stuart.** The structure of monoacetone *d*-xylulose, 607

Lichtman, A. L. Fatty acids and glucose in the blood of depancreatized dogs, 35

Link, Karl Paul. See CAMPBELL and LINK, 471

M

Mason, Harold L., Hoehn, Willard M., McKenzie, Bernard F., and **Kendall, Edward C.** Chemical studies of the suprarenal cortex. III. The structures of Compounds A, B, and H, 719

McKenzie, Bernard F. See MASON, HOEHN, MCKENZIE, and KENDALL, 719

Morgan, Agnes Fay, Kimmel, Louise, and **Hawkins, Nora C.** A comparison of the hypervitaminoses induced by irradiated ergosterol and fish liver oil concentrates, 85

Morgulis, Sergius. See SPENCER, MORGULIS, and WILDER, 257

Mueller, J. Howard. Nicotinic acid as a growth accessory for the diphtheria bacillus, 219

Murray, Sheila. See DEUEL, MURRAY, HALLMAN, and TYLER, 277

N

Neuwirth, Isaac. Sugar content of heparinized and oxalated plasmas, 463

Newton, Eleanor B. A chromogenic tungstate and its use in the determination of the uric acid of blood, 315

P

Pappenheimer, Alwin M., Jr. Diphtheria toxin. I. Isolation and characterization of a toxic protein from *Corynebacterium diphtheriæ* filtrates, 543

Price, J. Waide. See ROBINSON, PRICE, and HOGDEN, 481

R

Ravdin, I. S. See RIEGEL, RAVDIN, and ROSE, 523

Reiser, Raymond. The lipid analysis of human thoracic duct lymph, 625

Riegel, Cecilia, Ravdin, I. S., and **Rose, Henry J.** Effect of bile with and without cholesterol esters on esterification of cholesterol in blood plasma, 523

Rittenberg, D., Schoenheimer, Rudolf, and Evans, E. A., Jr. Deuterium as an indicator in the study of intermediary metabolism. X. The metabolism of butyric and caproic acids, 503

—. See SCHOENHEIMER and RITTENBERG, 155

Robinson, Howard W., Price, J. Waide, and Hogden, Corinne G. The estimation of albumin and globulin in blood serum. I. A study of the errors involved in the filtration procedure, 481

Rose, Henry J. See RIEGEL, RAVDIN, and ROSE, 523

Rothen, Alexandre. See LEVENE, ROTHEN, and KUNA, 759, 777

Rutenber, Charles B., and Andrews, James C. The applicability of the Benedict-Denis procedure to the determination of methionine sulfur, 203

S

Schmidt, E. G., Schmulovitz, M. J., Szczpinski, A., and Wylie, H. Boyd. The phenol and imidazole content of the blood, 705

Schmulovitz, M. J. See SCHMIDT, SCHMULOVITZ, SZCZPINSKI, and WYLIE, 705

Schoenheimer, Rudolf, and Johnston, Charles G. Lithocholic acid gallstones from hog bile, 499

Schoenheimer, Rudolf, and Rittenberg, D. Deuterium as an indicator in the study of intermediary metabolism. IX. The conversion of stearic acid into palmitic acid in the organism, 155

—. See RITTENBERG, SCHOENHEIMER, and EVANS, 503

Schroeder, E. F., and Woodward, Gladys E. The enzymatic hydrolysis of glutathione by rat kidney, 209

Schultze, M. O. See STÖTZ, HARRER, SCHULTZE, and KING, 129

Sendroy, Julius, Jr. Microdetermination of chloride in biological fluids, with solid silver iodate. I. Gasometric analysis, 335

—. II. Titrimetric analysis, 405

—. III. Colorimetric analysis, 419

—. Note on errors in the analysis of chloride in albuminous urine, 441

Shinohara, Kamenosuke. The determination of thiol and disulfide compounds, with special reference to cysteine and cystine. VIII. Molecular ratio between A-phospho-18-tungstic acid and cysteine in their color reaction, 743

Sidwell, A. E., Jr. See HOGNESS, SIDWELL, and ZSCHEILE, 239

Spencer, Howard C., Morgulis, Sergius, and Wilder, Violet M. A micromethod for the determination of gelatin and a study of the collagen content of muscles from normal and dystrophic rabbits, 257

Stekol, Jakob A., and **Hamill, William H.** On the non-labile deuterium of amino acids subjected to treatment in the medium of dilute deuterium oxide, 531

Stevens, Charles D. The source of the formic acid produced on acid hydrolysis of nucleic acids, 751

Stotz, Elmer, Harrer, Carter J., Schultze, M. O., and **King, C. G.** Tissue respiration studies on normal and scorbutic guinea pig liver and kidney, 129

Sullivan, M. X., and **Hess, W. C.** The effect of aldehydes on the quantitative determination of cysteine and cystine, 537

Szczpinski, A. See SCHMIDT, SCHMULOVITZ, SZCZPINSKI, and WYLIE, 705

T

Tipson, R. Stuart. A note on the acridine salts of "yeast" and "muscle" adenylic acids, 621

—. See LEVENE and TIPSON, 607

Toennies, Gerrit. Relations of thiourea, cysteine, and the corresponding disulfides, 297

Turner, C. W. See BERGMAN and TURNER, 21

—. See GRAHAM, HOUCHIN, and TURNER, 29

Tyler, David B. See DEUEL, MURRAY, HALLMAN, and TYLER, 277

V

Venning, Eleanor Hill, Evelyn, Kenneth A., Harkness, E. V., and **Browne, J. S. L.** The determination of estrin in urine with the photoelectric colorimeter, 225

du Vigneaud, Vincent. See BEHRENS and DU VIGNEAUD, 517

—. See JONES and DU VIGNEAUD, 11

W

Wilder, Violet M. See SPENCER, MORGULIS, and WILDER, 257

Woodward, Gladys E. See SCHROEDER and WOODWARD, 209

Wright, Norman. The infrared absorption spectra of the stereoisomers of cystine, 641

Wylie, H. Boyd. See SCHMIDT, SCHMULOVITZ, SZCZPINSKI, and WYLIE, 705

Y

Young, E. Gordon. On the separation and characterization of the proteins of egg white, 1

Young, Leslie. The effect of pyocyanine on the metabolism of cerebral cortex, 659

Z

Zscheile, F. P., Jr. See HOGNESS, SIDWELL, and ZSCHEILE, 239

INDEX TO SUBJECTS

A

Acidosis: Ammonia excretion (Alving and Gordon) 103
  Creatinine excretion (Alving and Gordon) 103
  Urea excretion (Alving and Gordon) 103

Acridine salts: Muscle adenylic acid (Tipson) 621
  Yeast adenylic acid (Tipson) 621

Adenylic acid: Muscle, acridine salts (Tipson) 621
  Yeast, acridine salts (Tipson) 621

Adrenal: Cortex compounds, structure (Mason, Hoehn, McKenzie, and Kendall) 719

Age: Brain protein amino acids, effect (Block) 467

Albumin: Blood serum, determination, filtration procedure, errors (Robinson, Price, and Hogden) 481

Aldehydes: Cysteine determination, effect (Sullivan and Hess) 537
  Cystine determination, effect (Sullivan and Hess) 537

Aldehydo tetraacetylmethyl-*d*-galacturonate: Synthesis (Campbell and Link) 471

Alkaloids: Ergot (Jacobs and Gould) 141
  Veratrine (Jacobs and Craig) 447

Amandin: Osmotic pressure, molecular weight, and stability (Burk) 63

Amines: Configurationally related, rotatory dispersion (Levene, Rothen, and Kuna) 759

Amino acid(s): α-, synthetic, subcutaneous injection, fate (Leighty and Corley) 331
  Brain proteins, age effect (Block) 467
  Catabolism (Leighty and Corley) 331
  Deuterium oxide-treated, nonlabile deuterium (Stekol and Hamill) 531
  Metabolism (Butts, Blunden, and Dunn) 289

Ammonia: Excretion, acidosis (Alving and Gordon) 103

Anserine: *l*-1-Methylhistidine synthesis (Behrens and du Vigneaud) 517

Antihemorrhage: Vitamin (Almquist) 635

Apparatus: Colorimeter, photoelectric, urine estrin determination (Venning, Evelyn, Harkness, and Browne) 225

I. A. R. I.

B

Bile: Blood plasma cholesterol esterification, effect (Riegel, Ravdin, and Rose) 523
  Hog, lithocholic acid gallstones (Schoenheimer and Johnston) 499
Biochemical reactions: Reaction velocity (Bodansky) 555
Blood: Lipid metabolism, depancreatized dog with insulin, choline effect (Kaplan and Chaikoff) 647
Bone: Sodium (Harrison) 457
Brain: Proteins, amino acids, age effect (Block) 467
Butyric acid: Metabolism, deuterium as indicator (Rittenberg, Schoenheimer, and Evans) 503

Calcified material: Sodium (Harrison) 457
Cannizzaro reaction: Catalytically induced reaction resembling (Levene and Christman) 575
Caproic acid: Metabolism, deuterium as indicator (Rittenberg, Schoenheimer, and Evans) 503
Catalytic reaction: (Levene and Christman) 575
Cerebrum: Cortex metabolism, pyocyanine effect (Young) 659
Cevine: Degradation products, basic (Jacobs and Craig) 447
Chloride: Biological fluids, colorimetric microdetermination, silver iodate (Sendroy) 419
  — —, gasometric microdetermination, silver iodate (Sendroy) 335
  — —, titrimetric microdetermination, silver iodate (Sendroy) 405
  Urine, albuminous, analysis, errors (Sendroy) 441
Cholesterol: Blood plasma, esterification, bile effect (Riegel, Ravdin, and Rose) 523
Choline: Ketonuria, effect (Deuel, Murray, Hallman, and Tyler) 277
  Lipid metabolism, blood and liver, depancreatized dog with insulin, effect (Kaplan and Chaikoff) 647
Collagen: Muscle, normal and dystrophic (Spencer, Morgulis, and Wilder) 257
Corynebacterium diphtheriæ: *See* Diphtheria bacillus
Creatinine: Excretion, acidosis (Alving and Gordon) 103
Cysteine: Determination (Shinohara) 743
  —, aldehyde effect (Sullivan and Hess) 537
  A-Phospho-18-tungstic acid and, color reaction, molecular ratio (Shinohara) 743
  Thiourea and corresponding disulfides, relations (Toennies) 297

Cystine: Determination (Shinohara) 743
—, aldehyde effect (Sullivan and Hess) 537
Hexo-, physiological availability (Jones and du Vigneaud) 11
—, synthesis (Jones and du Vigneaud) 11
Stereoisomers, infra-red absorption spectra (Wright) 641

D

Dentin: Constitution (Armstrong and Brekhus) 677
Deuterium: Butyric acid metabolism, deuterium as indicator (Rittenberg, Schoenheimer, and Evans) 503
Caproic acid metabolism, deuterium as indicator (Rittenberg, Schoenheimer, and Evans) 503
Metabolism, intermediary, indicator (Schoenheimer and Rittenberg) 155
(Rittenberg, Schoenheimer, and Evans) 503
Non-labile, amino acids treated with deuterium oxide (Stekol and Hamill) 531
Stearic acid conversion to palmitic acid, indicator (Schoenheimer and Rittenberg) 155
Diphtheria bacillus: Nicotinic acid as growth accessory (Mueller) 219
Toxic protein from filtrates (Pappenheimer) 543
Disulfide compounds: Determination (Shinohara) 743
Disulfides: Cysteine, relation (Toennies) 297
Thiourea, relation (Toennies) 297
Dopa: Formation from tyrosine, ultraviolet radiation (Arnow) 151

E

Egg: White proteins, separation and characterization (Young) 1
Electrolytes: Muscle dystrophy (Fenn and Goettsch) 41
Enamel: Constitution (Armstrong and Brekhus) 677
Enzyme(s): Kidney, glutathione hydrolysis (Schroeder and Woodward) 209
See also Phosphatase
Ergosterol: Irradiated, fish liver oil concentrates and, hypervitaminoses, comparison (Morgan, Kimmel, and Hawkins) 85
Ergot: Alkaloids (Jacobs and Gould) 141
Estrin: Urine, determination, photoelectric colorimeter (Venning, Evelyn, Harkness, and Browne) 225
Ethyl alcohol: Body storage, equilibrium (Harger, Hulpieu, and Lamb) 689
Excelsin: Osmotic pressure, molecular weight, and stability (Burk) 63

Fatty acids: Blood, depancreatized dogs (Lichtman) 35

Feces: Porphyrin excretion (DOBRINER) 115

Fish liver oil: Concentrates, irradiated ergosterol and, hypervitaminoses, comparison (MORGAN, KIMMEL, and HAWKINS) 85

Formic acid: Nucleic acid hydrolysis, source (STEVENS) 751

G

Galacturonate: Aldehydo tetraacetylmethyl-*d*-, synthesis (CAMPBELL and LINK) 471

Galacturonic acid: *d*-, mercaptal, synthesis (CAMPBELL and LINK) 471

Galacturonide: α-Methyl-*d*-, and derivatives, ring structure (LEVENE and KREIDER) 597

Poly-, methyl ester, oxidation and hydrolysis to levo-tartaric acid (LEVENE and KREIDER) 591

Gallstones: Hog bile, lithocholic acid (SCHOENHEIMER and JOHNSTON) 499

Gelatin: Microdetermination (SPENCER, MORGULIS, and WILDER) 257

Globulin: Blood serum, determination, filtration procedure, errors (ROBINSON, PRICE, and HOGDEN) 481

Glucose: Blood, depancreatized dogs (LICHTMAN) 35

—, determination, photoelectric (HOFFMAN) 51

Urine, determination, photoelectric (HOFFMAN) 51

Glutathione: Kidney enzyme, hydrolysis (SCHROEDER and WOODWARD) 209

Glycogen: Muscle, resynthesis, hexosemonophosphate (CORI, CORI, and HEGNAUER) 193

H

Hemoglobin: Derivatives, nitrite reactions (BARNARD) 177

Hemorrhage: Anti-, vitamin (ALMQUIST) 635

Hexocystine: Physiological availability (JONES and DU VIGNEAUD) 11

Synthesis (JONES and DU VIGNEAUD) 11

Hexomethionine: Physiological availability (JONES and DU VIGNEAUD) 11

Synthesis (JONES and DU VIGNEAUD) 11

Hexosemonophosphate: Muscle glycogen resynthesis from (CORI, CORI, and HEGNAUER) 193

Histidine: *l*-1-Methyl-, anserine synthesis from (BEHRENS and DU VIGNEAUD) 517

Hypervitaminosis: Ergosterol, irradiated, fish liver oil concentrates and, comparison (MORGAN, KIMMEL, and HAWKINS) 85

I

Imidazoles: Blood (SCHMIDT, SCHMULOVITZ, SZCZPINSKI, and WYLIE) 705

Insulin: Lipid metabolism, blood

and liver, depancreatized dog, choline and, effect (Kaplan and Chaikoff) 647

Isoleucine: *dl*-, fate (Butts, Blunden, and Dunn) 289

K

Ketonuria: Choline effect (Deuel, Murray, Hallman, and Tyler) 277

Ketosis: (Deuel, Murray, Hallman, and Tyler) 277

Kidney: Enzyme, glutathione hydrolysis (Schroeder and Woodward) 209

Respiration, normal and scorbutic animals (Stotz, Harrer, Schultze, and King) 129

L

Lactogenic hormone: Milk composition, effect (Bergman and Turner) 21

Leucine: *dl*-, fate (Butts, Blunden, and Dunn) 289

dl-Iso-, fate (Butts, Blunden, and Dunn) 289

dl-Nor-, fate (Butts, Blunden, and Dunn) 289

Lipid(s): Metabolism, blood and liver, depancreatized dog with insulin, choline effect (Kaplan and Chaikoff) 647

Thoracic duct lymph (Reiser) 625

Lithocholic acid: Gallstones, hog bile (Schoenheimer and Johnston) 499

Liver: Lipid metabolism, depancreatized dog with insulin, choline effect (Kaplan and Chaikoff) 647

Respiration, normal and scorbutic animals (Stotz, Harrer, Schultze, and King) 129

Lymph: Thoracic duct, lipids (Reiser) 625

Lysergic acid: -Related substances, synthesis (Jacobs and Gould) 141

M

Mammary gland: Urea production (Graham, Houchin, and Turner) 29

Metabolism: Intermediary, deuterium as indicator (Schoenheimer and Rittenberg) 155

(Rittenberg, Schoenheimer, and Evans) 503

Lipid, blood and liver, depancreatized dog with insulin, choline effect (Kaplan and Chaikoff) 647

Methionine: Hexo-, physiological availability (Jones and du Vigneaud) 11

—, synthesis (Jones and du Vigneaud) 11

Sulfur determination, Benedict-Denis method (Rutenber and Andrews) 203

Methyl-*d*-galacturonide: α-, and derivatives, ring structure (Levene and Kreider) 597

Methylhistidine: *l*-1-, anserine synthesis from (Behrens and du Vigneaud) 517

Milk: Lactogenic hormone stimulation, effect (BERGMAN and TURNER) 21
Monoacetone *d*-xylulose: Structure (LEVENE and TIPSON) 607
Muscle: Adenylic acid, acridine salts (TIPSON) 621
Collagen, normal and dystrophic (SPENCER, MORGULIS, and WILDER) 257
Dystrophy, electrolytes (FENN and GOETTSCH) 41
Glycogen, resynthesis, hexosemonophosphate (CORI, CORI, and HEGNAUER) 193

N

Neuroproteins: Chemistry (BLOCK) 461
Nicotinic acid: Diphtheria bacillus growth accessory (MUELLER) 219
Norleucine: *dl*-, fate (BUTTS, BLUNDEN, and DUNN) 289
Nucleic acids: Hydrolysis, acid, formic acid source (STEVENS) 751

O

Oil: *See also* Fish liver oil

P

Palmitic acid: Stearic acid conversion, deuterium as indicator (SCHOENHEIMER and RITTENBERG) 155
Pancreatectomy: Blood fatty acids, effect (LICHTMAN) 35
— glucose, effect (LICHTMAN) 35
Pancreatectomy—*continued:*
Lipid metabolism, blood and liver, choline and insulin effect (KAPLAN and CHAIKOFF) 647
Phenols: Blood (SCHMIDT, SCHMULOVITZ, SZCZPINSKI, and WYLIE) 705
Phosphatase: Blood serum, activity, determination (BODANSKY) 167
Phosphate: Inorganic, blood serum, determination (BODANSKY) 167
Polygalacturonide: Methyl ester, oxidation and hydrolysis to levo-tartaric acid (LEVENE and KREIDER) 591
Porphyrin: Excretion, feces (DOBRINER) 115
Potassium: Blood serum, microdetermination, photoelectric (HOFFMAN) 57
Protein(s): Brain, amino acids, age effect (BLOCK) 467
Egg white, separation and characterization (YOUNG) 1
Neuro-, chemistry (BLOCK) 467
Osmotic pressure, molecular weight, and stability (BURK) 63
Toxic, diphtheria bacillus filtrates (PAPPENHEIMER) 543
Pyocyanine: Cerebral cortex metabolism, effect (YOUNG) 659

R

Reaction velocity: Biochemical reactions (BODANSKY) 555

S

Scurvy: Kidney respiration (Stotz, Harrer, Schultze, and King) 129
  Liver respiration (Stotz, Harrer, Schultze, and King) 129
Sodium: Bone (Harrison) 457
  Calcified material (Harrison) 457
Stearic acid: Palmitic acid, conversion, deuterium as indicator (Schoenheimer and Rittenberg) 155
Sterol: -Related compounds, absorption spectra (Hogness, Sidwell, and Zscheile) 239
Substitution reaction: Walden inversion and, mechanism (Levene, Rothen, and Kuna) 777
Sugar: Blood plasma, heparinized and oxalated (Neuwirth) 463
Sulfur: Methionine, determination, Benedict-Denis method (Rutenber and Andrews) 203
Suprarenal: *See* Adrenal

T

Tartaric acid: Levo-, polygalacturonide methyl ester oxidation and hydrolysis (Levene and Kreider) 591
Thiocyanate: Tissues, determination (Brodie and Friedman) 511
Thiol compounds: Determination (Shinohara) 743
Thiourea: Cysteine and corresponding disulfides, relations (Toennies) 297
Thoracic duct: Lymph, lipids (Reiser) 625
Tissue: Respiration, normal and scorbutic animals (Stotz, Harrer, Schultze, and King) 129
Tungstate: Blood uric acid determination (Newton) 315
Tyrosine: Dopa formation from, ultraviolet radiation (Arnow) 151

U

Ultrafiltration: Anaerobic (Lavietes) 267
Urea: Excretion, acidosis (Alving and Gordon) 103
  Mammary gland, production (Graham, Houchin, and Turner) 29
  Thio-, cysteine, and corresponding disulfides, relations (Toennies) 297
Uric acid: Blood, determination (Newton) 315
Urine: Albuminous, chloride analysis, errors (Sendroy) 441
  Estrin determination, photoelectric colorimeter (Venning, Evelyn, Harkness, and Browne) 225

V

Veratrine: Alkaloids (Jacobs and Craig) 447

Vitamin: Antihemorrhagic (Almquist) 635

W

Walden inversion: Substitution reaction and, mechanism (Levene, Rothen, and Kuna) 777

X

Xylulose: *d*-, monoacetone, structure (Levene and Tipson) 607

Y

Yeast: Adenylic acid, acridine salts (Tipson) 621